STUDY OF STARCH DEBRANCHING ENZYMES IN DEVELOPING WHEAT KERNELS

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ABSTRACT

Starch debranching enzymes, which specifically hydrolyse α-1,6-glucosidic bonds in glucans containing both α-1,4 and α-1,6 linkages, are classified into two types: isoamylase (EC. 3.2.1.68) and limit dextrinase (EC. 3.2.1.41). The starch debranching enzymes are primarily involved in starch degradation during seed germination. Recently, however, starch debranching enzymes, particularly isoamylase, have been implicated in starch biosynthesis in many plant species.

In this study, a 2,590-bp isoamylase cDNA, which encoded an 88-kDa isoamylase pre-protein containing the N-terminal transit peptide of 5 kDa, was isolated from a developing wheat kernel cDNA library. The recombinant protein produced in *E. coli* by expression of the cDNA exhibited isoamylase-type debranching enzyme activity. Accumulation of the 2.6-kb isoamylase transcripts in various tissues of wheat indicated the involvement of isoamylase in both starch synthesis in storage and non-storage organs, and starch degradation during seed germination. In the early stages during wheat kernel development, the presence of a 1.7-kb transcript was observed. The 1.7-kb transcript encoded a 51-kDa truncated isoamylase, whose starch debranching enzyme activity could not be detected *in vitro*. Southern blot analysis of genomic DNA of hexaploid wheat (*Triticum aestivum* L. cultivar CDC Teal) showed that isoamylase gene existed as a single copy in the wheat genome. Therefore, the production of various isoamylase transcripts and the involvement of isoamylase during two developmental stages of wheat plant may result from transcriptional

modification of the existing gene and/or expression from a distantly related gene.

Western blot analysis using rabbit antibodies raised against wheat isoamylase detected a single polypeptide of 83-kDa from developing wheat kernels. Accumulation of isoamylase polypeptide increased as the kernels developed from five to 15 days post-anthesis (dpa), and then started to decrease in 20-dpa kernels until it could not be detected in mature kernels. Similar to the presence of isoamylase, highest activity of limit dextrinase was observed in 15-dpa wheat kernels. According to the amounts of polysaccharides present at different stages during wheat kernel development, the concentration of starch increased as kernels matured, while the amylose/amylopectin ratio remained constant until the kernels reached a fully matured stage. The relationships between the concentrations of polysaccharides and the presence of isoamylase polypeptide, and the activity of limit dextrinase in developing wheat grains have suggested that debranching activities of starch debranching enzymes are essential to maintain a constant ratio of amylose and amylopectin during wheat kernel development.

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To my loving parents

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LIST OF ABBREVIATIONS

AGPase	ADP-glucose pyrophosphorylase
bp	Base pairs
cDNA	Complementary deoxyribonucleic acid
Ci	Curie
Con A	Concanavalin A
CTAB	Cetyltrimethylammonium bromide
cv	Cultivar
DEPC	Diethyl pyrocarbonate
DIG	Digoxigenin
DMSO	Dimethyl sulfoxide
dNTPs	Deoxynucleotide triphosphates
dpa	Days post-anthesis
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
GBSS	Granule-bound starch synthase
IPTG	Isopropyl-β-D-thiogalactopyranoside
kb	Kilobase
kDa	Kilodalton
LB	Luria Bertani
MOPS	3-(N-morpholino)propanesulfonic acid
mRNA	Messenger ribonucleic acid
PCR	Polymerase chain reaction
pfu	Plaque-forming units
RT-PCR	Reverse transcriptase polymerase chain reaction
RACE	Rapid amplification of cDNA ends
rRNA	Ribosomal ribonucleic acid
SBE	Starch branching enzyme
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SS	Starch synthase
Tris	Tris(hydroxymethyl)aminomethane

1. INTRODUCTION

Starch, a reserve carbohydrate in plants, is present in storage organs such as seeds and tubers. Three major cereals (wheat, rice, and maize) along with three tuber crops (potato, cassava, and yam) provide four-fifths of the calories required by human beings (Mauro, 1996). Despite the use in food products, starch has also been sought as an environmentally friendly industrial polymer. Both the dietary and industrial uses of starch have expanded, thus creating an increased demand for starch in the market place.

Starch from various botanical sources has different properties, and thus is suitable for different end uses. However, there are limitations in the physicochemical properties of natural starch, which often make it unsuitable for the demands of industrial processing. The problems derive largely from the instability of solution and paste viscosity to changes in temperature, and pH. These problems can be overcome by chemical modification of starches (McCleary, 1980). For example, dialdehyde starch derived from oxidization of starch with sodium hypochlorite is useful in the paper and textile industries, while cross-linked starch is used in food products where extreme temperature and/or pH processing is applied (Koch and Roper, 1988). Large-scale production of a chemically modified starch requires several steps of treatment including purification of the modified products from other contaminants.

Therefore, modification of starch usually results in a high production cost. Alternatively, it would be economically valuable to produce plants with starch properties suitable for a specific use. This can be accomplished using traditional plant breeding. However, this process is time and labor consuming, and does not ensure that plants with a desired trait will be obtained.

Introduction of genetic engineering has given new insight into the development of plant lines with different starch properties. This can be accomplished within a shorter period of time as compared to a breeding program (Jauhar, 2001). To efficiently apply the techniques of genetic engineering, the starch synthesis pathway must be clearly understood. Three enzymes (ADP-glucose pyrophosphorylase, starch synthase, and starch branching enzyme) are known to participate in the starch synthesis pathway. These enzymes have been characterized in wheat and in many other plant species.

Starch debranching enzymes, which specifically hydrolyse α-1,6-glucosidic bonds of starch, are primarily involved in starch degradation during seed germination (Steup, 1988). Based on substrate specificity, starch debranching enzymes are divided into two classes, limit dextrinase and isoamylase. The lack of starch debranching enzyme activities in some species results in accumulation of phytoglycogen and low production of starch in storage organs (Pan and Nelson, 1984, Nakamura et al., 1996b) or leaves (Zeeman et al., 1998) of mutant lines. These observations have therefore implicated a role of starch debranching enzymes in starch biosynthesis.

Given the importance of wheat in Western Canada, the objective of this project was to study the involvement of starch debranching enzymes in wheat starch synthesis. The experiments conducted to accomplish this goal were as follows:

1. Limit dextrinase activities in different stages of developing wheat kernels were determined.
2. Due to unavailability of a specific substrate, isoamylase activity could not be determined from plant extracts. Therefore, an isoamylase cDNA was isolated from developing wheat kernels. A recombinant protein encoded by the isolated cDNA was produced in an *E. coli* expression vector, and then used to produce polyclonal antibodies in rabbits. The antibodies were used to determine the presence of isoamylase polypeptide in different stages of developing wheat kernels.
3. Results obtained from the above were correlated with the concentrations of starch, amylose, and amylopectin present in developing wheat grains. The possible involvement of limit dextrinase and isoamylase in wheat starch synthesis was discussed.

2. LITERATURE REVIEW

2.1 Wheat

Wheat is a member of the tribe *Triticeae* in the grass family *Gramineae*, which produces dry, one-seeded fruit, normally called a kernel or grain. The wheat kernel develops within a floral envelope, which consists of modified leaves. The kernel (Figure 2.1) is covered with many cell layers: the pericarp (fruit coat), testa (seed coat), and aleurone layer, arranged from the outermost to the innermost layer, respectively. These layers, collectively known as bran, protect the components inside the kernel. The starchy endosperm, which represents about 80% of the kernel weight, contains starch granules and storage proteins. The embryo, which comprises 2-3% of the wheat kernel, is composed of two major parts: the embryonic axis (rudimentary root and shoot) and the scutellum (Hoseney, 1994).

Wheat (genus *Triticum*) comprises a series of diploid (e.g. *T. monococcum*), tetraploid (e.g. *T. turgidum*), and, more commonly, hexaploid forms (*T. aestivum*). These species can be further subclassified on the basis of color (white or red) or hardness (soft or hard) of the grain or growth habit (spring or winter) (Oleson, 1994). According to these characteristics, wheats are generally divided into seven classes: Hard Red Spring wheat, Hard Red Winter wheat, Soft Red Winter wheat, Durum wheat, White wheat, Unclassed wheat, and Mixed wheat (Faridi and Faubion, 1995). Based on characteristics and end

uses of the grains, Canadian Wheat Board has specifically classified Canadian wheats into seven market classes: Canada Western Red Spring, Canada Western Amber Durum, Canada Prairie Spring Red, Canada Prairie Spring White, Canada Western Red Winter, Canada Western Extra Strong, and Canada Western Soft White Spring. Each type of wheat has different properties, and thus is suitable for different end-uses (Morris and Rose, 1996). For example, Hard Red Winter and Hard Red Spring wheat are suitable for yeast-leavened bread, while Soft Red Winter and common White wheat are primarily used for pastries, crackers, and cookies.

Due to highly repetitive DNA sequences, wheat has a relatively large genome (1.7×10^{10} bp) as compared to other cereals (Keller and Feuillet, 2000). The polyploid characteristic of modern wheat has arisen by exchange of genetic materials between *Triticum* species and diploid species of the genus *Aegilops*. For example, hexaploid bread wheat arose through spontaneous hybridization of the diploid species *Triticum urartu* (AA genome) and unknown species (BB genome) to form tetraploid wheat (AABB). Further hybridization with *Aegilops tauschii* (DD) led to production of commercial bread wheat (*Triticum aestivum*, 2n = 6x = 42; AABBDD) (Devos and Gale, 2000). The wheat genomes have highly similar gene contents and orders (Chao et al., 1989). Therefore, most genes exist in triplicated homoeologous sets, one from each genome. Due to random mutations that occur along genomes, however, the alleles isolated from different genomes are not necessarily identical.

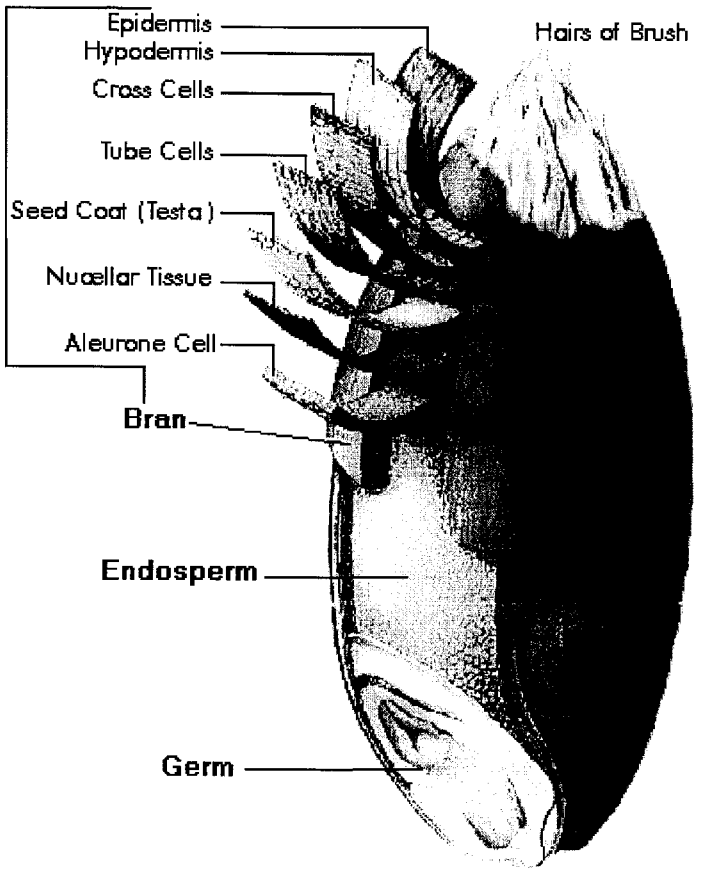


Figure 2.1 Wheat kernel
The figure shows the longitudinal section of a wheat kernel. Cell layers comprising the kernel are indicated.
(Source: http://www.crop.cri.nz/fooodinfo/millbake/whgrain.htm)

2.2 Starch and starch granules

Starch is the major reserve carbohydrate in plants. It is present in practically every type of tissue: leaf, fruit, root, shoot, stem, pollen, and seed. In cereal grains, starch is the primary source of stored energy. The amount of starch contained in cereal grains varies depending on species, and developmental stages. However, it generally ranges between 60 and 75% of the weight of the grains and provides 70-80% of the calories consumed by humans worldwide (Thomas and Atwell, 1999). Starch is synthesized either as a transient molecule in chloroplasts of leaves or as a reserve polysaccharide in amyloplasts of storage organs. In leaves, transient accumulation of starch takes place during the light period and is mobilized as sucrose transported to various non-photosynthetic organs during the dark period (Martin and Smith, 1995). Starch in plant storage organs, e.g. seeds and tubers, is synthesized and stored in the form of water insoluble granules.

Starch granules from different botanical origins vary in size, shape, and structure. The diameters of the granules can range from less than 1 μm to more than 100 μm, and the shape may be spherical, ovoid, or lenticular (Banks and Muir, 1980). In wheat, barley, rye, and triticale, two types of starch granules are found in the endosperm (Stark and Lynn, 1992). The large (A-type) starch granules of wheat are disk-like or lenticular in shape, with an average diameter of 10 - 35 μm, whereas the small (B-type) starch granules are roughly spherical or polygonal in shape, ranging from 1 to 10 μm in diameter. (Evers, 1973, Stark and Lynn, 1992, Peng et al., 1999). A-type starch granules contribute > 70% of

the total weight, and about 3% of the total granule number of endosperm starch. On the other hand, B-type starch granules account for > 90% of the total granule number, but < 30% of the total weight of starch in wheat endosperm (Evers 1973, Morrison and Gadan 1987).

Partially degraded starch granules from storage organs reveal a unique internal structure under a scanning electron microscope. Granules exhaustively treated with acid and enzyme demonstrate a growth ring pattern (Figure 2.2) (Buléon et al., 1998). This pattern results from uneven degradation between semi-crystalline and amorphous regions; the amorphous growth rings are more sensitive to chemical and enzymatic attack. The semi-crystalline growth rings are composed of alternating crystalline and amorphous lamellae. The size of one amorphous and one crystalline lamella layer (9 nm) is highly conserved throughout the plant kingdom (Jenkins et al., 1993). The crystalline lamellae contain tightly packed double helices of parallel α-1,4 glucan chains of amylopectin, while a high density of the α-1,6-branches is located in the amorphous lamellae. A large portion of amylose is found within the amorphous growth rings, with only small amounts co-crystallized with amylopectin within crystalline lamellae (Kasemsuwan and Jane, 1994). The growth ring configuration is believed to result from alternating periods of growth and rest when starch is synthesized at a higher and lower rate, respectively (French, 1984). Buttrose (1962) reported that the growth ring pattern of wheat starch granules was observed only when plants were subjected to diurnal illumination.

A.

B.

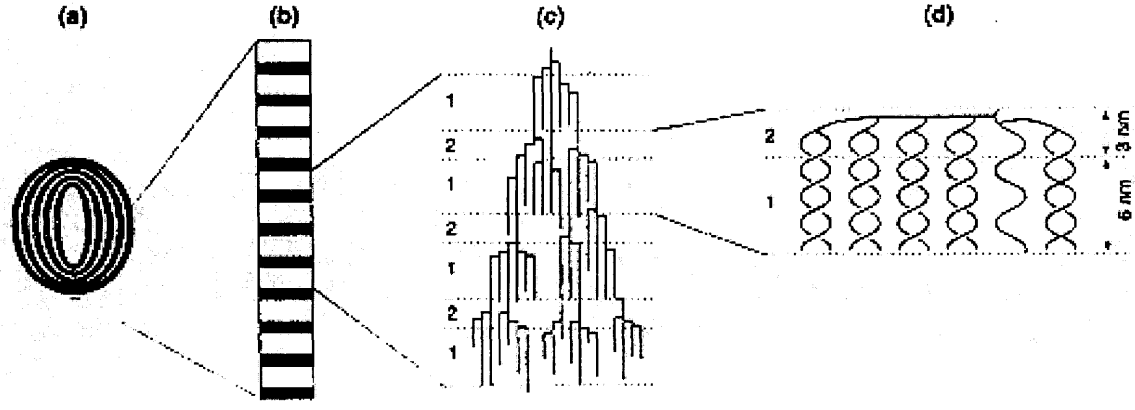


Figure 2.2 Starch granule growth rings

A. Cross section of sorghum kernels treated with α-amylase (Thomas and Atwell, 1999). The starch granule growth rings are shown. The scale bar (10 μm) is located on the upper right corner.

B. Schematic view of a starch granule showing a succession of amorphous and semi-crystalline growth rings (a). The semicrystalline growth ring (b) is composed of alternating crystalline and amorphous lamellae as shown in white and black regions, respectively. Amylopectin molecule is arranged in the manner that the α-1,6-linkages are located in amorphous lamellae and short α-1,4-glucan chains are packed in crystalline lamellae (c). A single layer of amorphous and crystalline lamella (9 nm) is shown in (d) in which the parallel α-1,4 linked glucan chains form double helix structures (Ball et al., 1998).

Starch granules from storage organs and leaves have rather different macrostructures. Biosynthesis and degradation of leaf starch are highly dynamic; therefore, limited information on leaf starch granules is currently available. Starch granules in leaves are generally smaller than those found in storage tissues. Unlike those in storage organs, the shape of leaf starch granules is not species specific and is likely to be determined simply by the space available at the site where they are formed (Preiss and Sivak, 1996).

2.3 Composition of starch granules

The starch granule is mainly composed of two polysaccharides, amylose and amylopectin, arranged into a three-dimensional, semicrystalline structure. The building block of both amylose and amylopectin is D-glucopyranose. However, the biochemical properties of amylose and amylopectin are considerably different (Table 2-1). In addition to the polysaccharide components (~25% amylose and ~75% amylopectin), starch granules also contain other minor components (1%) such as protein, lipid and ash.

2.3.1 Amylose

Amylose is a predominantly linear chain of α-1,4-linked glucopyranose. Based on incomplete digestion with β-amylase, amylose contains sparse branches (approximately one branch per 1,000 glucose residues or 8-19 branch points per molecule) (Hizukuri et al., 1981, Takeda et al., 1987, Martin and Smith, 1995).

Table 2-1 Properties of amylose, amylopectin, and glycogen

	Amylose	***Amylopectin***	***Glycogen***
General structure	essentially unbranched	branched	highly branched
Branch (%)	0.2-1.0	4.0-5.5	8.0-10.0
Average chain length (C.L.)[1]	100-550	18-25	12-15
Degree of polymerization (D.P.)[2]	~ 10^3	10^5-10^6	~ 10^5
Iodine coloration	deep blue	purple	brown
λ_{max} ***(nm)***	~ 660	530-550	430-450
Stability of aqueous solution	retrograde	stable	stable
Conversion into maltose (%)			
With β-amylase	~70	~55	~45
With isoamylase and then β-amylase	≤ 99	~75	N/D[3]
With α-amylase	≤ 99	~90	~80

[1]*Chain length = the number of glucose residues per non-reducing end group*
[2]*Degree of polymerization = the number of glucose residues per reduction end group*
[3]*N/D = no data*
The table was modified from Nakamura (1996).

Amylose usually accounts for approximately 25% of starch weight. However, the amylose concentration in a particular starch sample can vary over a wide range. For example, amylose is entirely absent in *waxy* mutants of many plant species (Weatherwax, 1922, Murata et al., 1965, Nakamura et al., 1995, Delrue et al., 1992), whereas it accounts for 55% or higher in amylose *extender (ae)* (Banks and Greenwood, 1975) and *dull1* mutants of maize (Davis et al., 1955).

The long chain of α-1,4-linked glucose residues in amylose forms a helical structure consisting of six glucose residues per turn (Lewis, 1984). This structure allows amylose to form a clathrate complex with free fatty acids, alcohols, or iodine by locking the guest molecules within the interior of the helix (Thomas and Atwell, 1999). Interaction between amylose and iodine results in the formation of a blue complex. This simple method has been used to identify the presence of, and quantitate, amylose in a sample.

2.3.2 Amylopectin

Amylopectin is a highly branched molecule containing α-1,6 glucosidic bonds creating a branch point in the linear glucan chains. A chain of approximately 20 α-1,4-linked glucose residues is joined by α-1,6 linkages to other chains (Martin and Smith, 1995). Peat et al. (1952) classified short glucan chains in amylopectin molecules as A, B, and C chains. An A chain is linked to the molecule only by its potential reducing group. A B chain is similarly linked but also carries one or more A or B chains. A C chain is distinguished by the presence of the sole reducing group in the molecule. Hizukuri (1986) further

classified B-chains according to their lengths: B_1, B_2, and B_3 consist of 20-24, 42-48, and 69-75 glucose residues, respectively. Many models have been proposed to explain the arrangement of A, B, and C chains in the amylopectin molecule (Figure 2.3) (for review see Manners, 1985). The cluster model proposed by French (1973) explains the possibility of building the high molecular weight molecule (10^7-10^8) with short glucan chains of 20-25 glucose residues. According to French (1973), the molecular weight of amylopectin increases by simply adding the number of clusters. Robin et al. (1974) proposed a somewhat similar model, but noted that the terms A, B, and C chains in their model are different from those given by Peat et al. (1952). According to sequential enzymatic treatment of amylopectin, Robin et al. (1974) named the chains consisting of 20-25, 45, and 60 glucose residues as A, B, and C chains, respectively.

Inside starch granules, the short parallel α-1,4 glucan chains of amylopectin form a double helix structure (Figure 2.2B). However, a large number of branch points disrupt the formation of iodine-binding helices. In addition, the α-1,4 glucan chains of amylopectin are too short to form a stable complex with iodine. Therefore, only a small amount of iodine binds to amylopectin, resulting in the formation of a brownish colored complex. In addition to iodine, amylopectin can also form a complex with the lectin concanavalin A (Con A) (Matheson and Welsh, 1988). Under defined conditions of pH, temperature, and ionic strength, Con A specifically complexes branched polysaccharides based on α-D-glucopyranosyl or α-D-mannopyranosyl units at

multiple non-reducing groups with the formation of a precipitate (Colonna et al., 1985). Since Con A is unable to form a complex with amylose, the amount of amylose can be determined after treatment of a starch sample with Con A (Yun and Matheson, 1990).

2.3.3 Minor components of starch granules

Protein, lipid, and ash (minerals and salts) are present in starch granules in very small quantities. The protein content of starch varies from 0.05 to 0.5% depending on the botanical source. Starch granule proteins have been divided into two types based on the ability to be extracted from the granules (Rayas-Duarte et al., 1995). Surface starch granule proteins can be extracted with salt solution, whereas integral starch granule proteins require more rigorous extraction methods (e.g. with sodium dodecyl sulfate [SDS]). It is believed that the integral proteins are covalently bound to the amylose-amylopectin structure inside the granule, while the surface proteins are loosely associated with the exterior of the granule.

Cereal starches generally contain a relatively high percentage of fatty substances (0.6-1.0%) as compared to potato (0.05%) and tapioca (0.1%) starch. The main lipids in wheat starch are phosphorus-containing lipids called lysophospholipids, whereas free fatty acids are the main lipid component in maize starch. In maize and wheat starch granules, at least a portion of the lipids exists as an amylose-lipid inclusion complex (Swinkels, 1985).

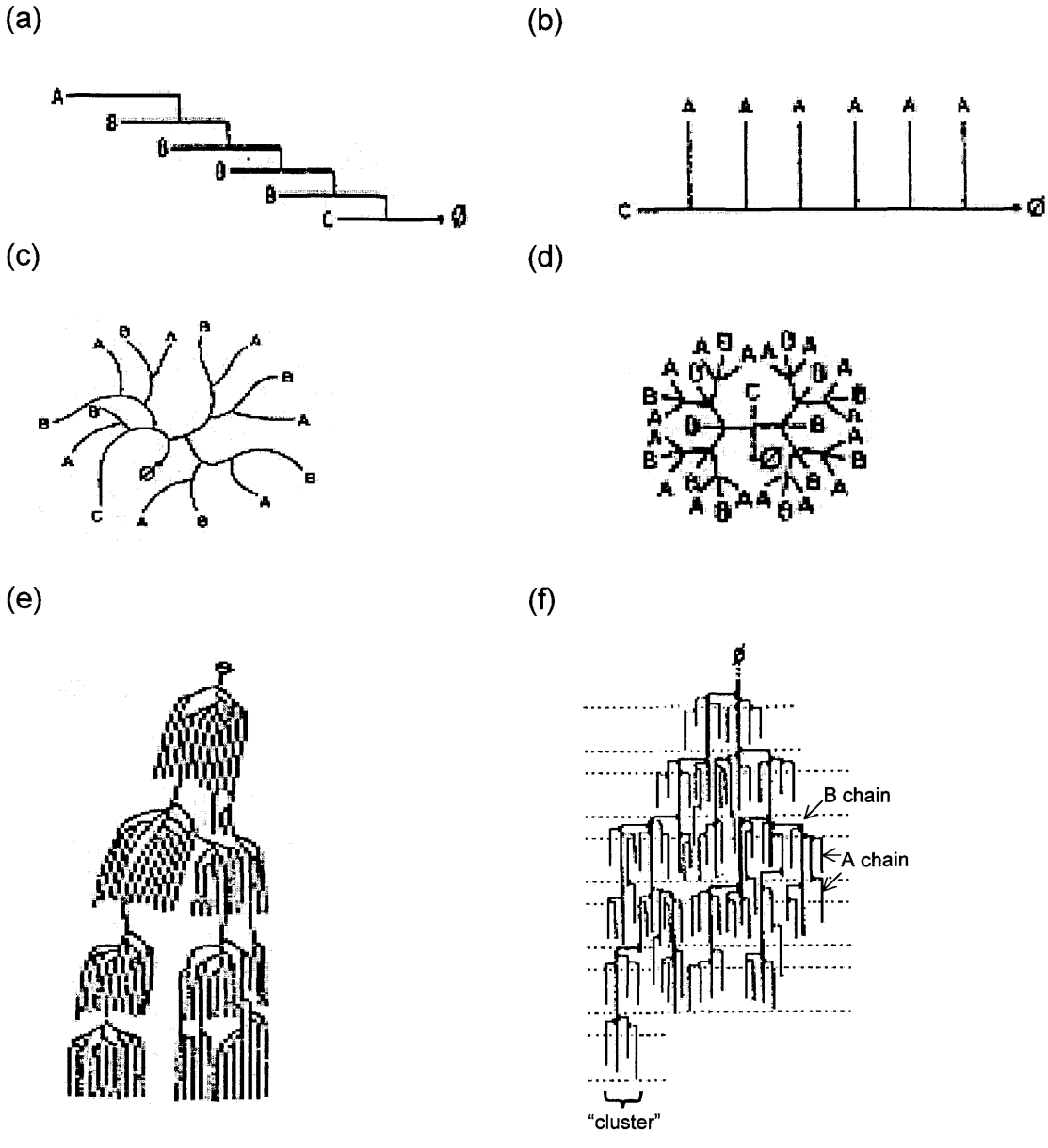


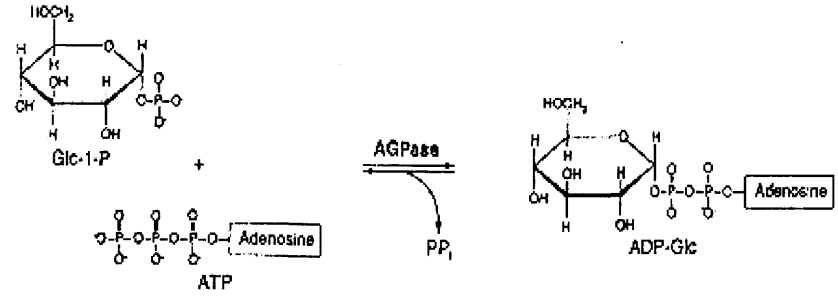
Figure 2.3 Structural models of amylopectin
Diagrammatic representations of the molecular structure of amylopectin as proposed by (a) Haworth, (b) Staudinger, (c) Meyer, (d) Meyer (revised structure), (e) French, and (f) Robin. A, B, and C indicate different types of glucan chains as mentioned in the text. The symbol Ø indicates reducing ends of the molecules (Manners, 1985).

Ash content in starch granules varies depending on plant species; however, it is generally less than 0.5% (dry basis) (Thomas and Atwell, 1999). Potato starch has a relatively high ash content due to the presence of phosphate groups (0.06-0.10%, calculated as P). The negatively-charged phosphate groups in potato starch occur as monoesters (about 1 phosphate monoester group per 300 glucose units) that are linked to the C-6 (~70%) and C-3 (~30%) positions of the glucose residues in amylopectin molecules (Takeda and Hizukuri, 1982).

2.4 Starch synthetic enzymes

It is widely accepted that three major enzymes catalyze starch synthesis in plants (Figure 2.4). The first enzyme, ADP-glucose pyrophosphorylase, catalyzes the formation of ADP-glucose, the substrate for starch biogenesis. Starch synthase catalyzes the elongation of glucan chains by introduction of α-1,4-glucosidic linkage between the incoming glucose residue and the growing glucan chain. Starch branching enzyme introduces α-1,6-glucosidic bonds to form branched polysaccharides. Various isoforms of these enzymes have been identified in many plant species. Different catalytic activities carried by each isoform have indicated distinct roles of the enzyme isoforms in starch biosynthesis. Recently, starch degrading enzymes, for example starch debranching enzymes (Myers et al., 2000, Smith, 1999) and disproportionating enzymes (Colleoni et al., 1999b), have also been implicated in starch biosynthesis pathway. The involvement of starch debranching enzymes in starch biosynthesis will be discussed in section 2.8.

A.



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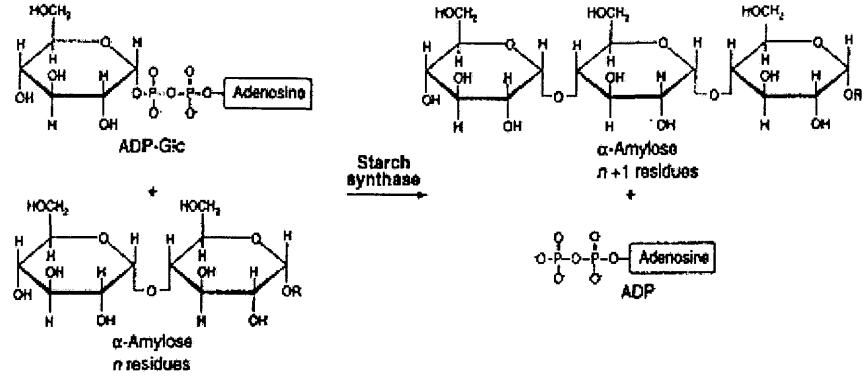

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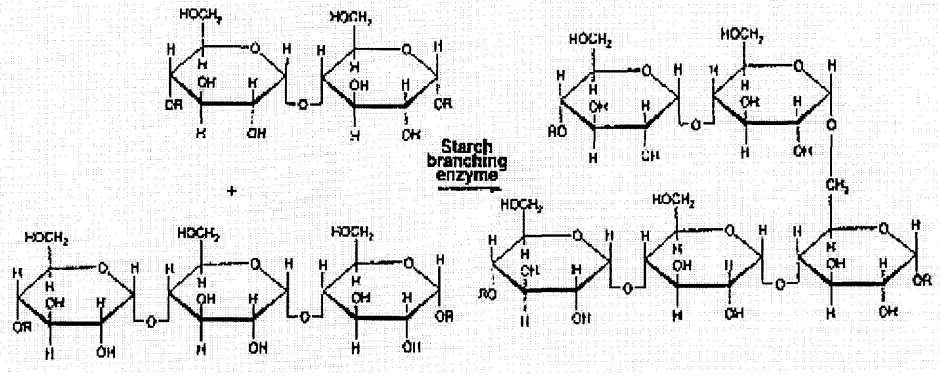


Figure 2.4 Reactions catalyzed by starch synthetic enzymes
The diagram shows three major steps in starch biosynthesis pathway. (A) ADP-glucose pyrophosphorylase (AGPase) catalyzes the formation of ADP-glucose (ADP-glc). (B) Starch synthase adds a glucose unit to the non-reducing end of a glucan chain. (C) Starch branching enzyme introduces an α-1,6-glucosidic bond to amylopectin molecule (Slattery et al., 2000).

2.4.1 ADP-glucose pyrophosphorylases (ATP: α-glucose-1-phosphate adenylyl-transferases, or AGPases; EC 2.7.7.27)

AGPase catalyzes the initial step in starch synthesis in bacteria and plants. The enzyme is found in all plant tissues that contain starch granules. The relationship between the level or activity of AGPase and starch content has indicated that AGPase catalyzes the rate-limiting step in starch biosynthesis for both photosynthetic and non-photosynthetic tissues (Preiss, 1988). Both bacterial and plant enzymes are regulated by allosteric effectors. Most plant AGPases are activated by 3-phosphoglycerate (3-PGA) and inhibited by inorganic phosphate (P_i) (Preiss and Sivak, 1996). However, AGPases from developing pea (Hylton and Smith, 1992), and endosperm of barley (Kleczkowski et al., 1993), and wheat (Olive et al., 1989) show no or insignificant responses to 3-PGA or P_i. Unlike the homotetrameric structure of the bacterial enzyme which is encoded by a single gene, the plant AGPase exists as a tetramer (210-240 kDa) composed of two small (50-55 kDa) and two large (51-60 kDa) subunits, which are encoded by distinct genes (Kleczkowski et al., 1991, Smith-White and Preiss, 1992). It is believed that only the small subunits act as catalytic subunits, whereas the large subunits may have a strictly regulatory function (Ball and Preiss, 1994). In maize (Denyer et al., 1996) and barley (Thorbjørnsen et al., 1996), plastidal and cytosolic isoforms of AGPases have been identified, with the latter contributing dominant activity in the endosperm.

2.4.2 Starch synthases (SSs, or ADP-glucose glucosyl transferases; EC 2.4.1.21)

SSs occur in two main forms, one bound to the starch granules and the other solubilized in the stroma of chloroplasts and amyloplasts, thus namely granule-bound (GBSS) and soluble (SS) starch synthases, respectively.

2.4.2.1 Granule-bound starch synthase (GBSS)

Amylose free- (*waxy*) mutants, which have been identified in many plant species, lack the *waxy* protein of 58-60 kDa (Weatherwax, 1922, Murata et al., 1965, Nakamura et al., 1995, Delrue et al., 1992). The *waxy* protein, which is also known as GBSSI, is responsible for amylose synthesis in plant storage organs (Nelson and Rines, 1962). In wheat, GBSSI is encoded as 67-kDa pre-protein carrying a 7-kDa transit peptide at the N-terminus (Clark et al., 1991). Ainsworth et al. (1993) showed that the GBSSI gene in wheat exists as a triplicated set of single copy (one on each genome). All three *waxy* alleles of hexaploid wheat have recently been characterized (Murai et al., 1999, Yan et al., 2000). The lesions of the *waxy* mutation in wheat are highly specific to endosperm and pollen tissues (Vrinten and Nakamura, 2000), while the pericarp starch granules of *waxy* wheat lines show significantly higher GBSS activity than do the endosperm starch granules (Nakamura et al., 1998). High activity of GBSS present in pericarp tissue correlates with the presence of a 59-kDa protein associated with starch granules from pericarp but not those from endosperm (Nakamura et al., 1998). The 59-kDa protein is then designated as GBSSII. In addition to pericarp, GBSSII is also expressed in leaf, and culm

tissues. Therefore, Vrinten and Nakamura (2000) suggested a role of GBSSII in amylose synthesis in non-storage organs. Although the deduced amino acid sequences of GBSSI and GBSSII are 66% identical, the proteins are encoded by separate genes located on different chromosomes (Vrinten and Nakamura, 2000). Similar to the observation of GBSSII in hexaploid wheat, Fujita and Taira (1998) observed a 56-kDa granule-bound starch synthase, possibly GBSSII, in pericarp, aleurone layer, and embryo of immature seeds in diploid wheat (*T. monococcum*).

2.4.2.2 Soluble starch synthase (SSS)

Multiple starch synthases, which display different physical and kinetic properties, have been described in the soluble fraction of maize (Dang and Boyer, 1988), rice (Baba et al., 1993), wheat (Li et al., 1999a), pea (Denyer and Smith, 1992), potato (Edwards et al., 1995), and *Chlamydomonas reinhardtii* (Fontaine et al., 1993). Because these enzymes are partitioned between the granule and the soluble phase, they are generally referred to as starch synthases (SSs) rather than soluble starch synthases (SSSs). So far, three classes of SSs have been found in higher plants; type I (Li et al., 1999b), II (Craig et al., 1998, Imparl-Radosevich et al., 1999), and III (Gao et al., 1998, Li et al., 2000). SSI of wheat is partitioned between the granule and the soluble fraction (Li et al., 1999b, Peng et al., 2001). Wheat SSII is predominantly granule-bound with only a small amount present in the soluble fraction (Gao and Chibbar, 2000). SSIII is exclusively found in the soluble fraction of wheat endosperm (Li et al., 2000). The ratios of SS isoforms and/or their enzymatic

activities in various tissues are different. For example, most of the soluble starch synthase activity in pea leaf is contributed by SSIII, while the major isoform in pea embryo is SSII, which contributes only 15% in leaf (Tomlinson et al., 1998). In developing wheat endosperm, SS activity is mainly contributed by SSI (Peng et al., 2001). Distinct localization of SS isoforms may therefore indicate different roles of each isoform in amylopectin synthesis. A study of the mutations at the *rug5* locus of pea has suggested a role of SSII in the synthesis of amylopectin chains of intermediate length (B_2 and B_3 chains) (Craig et al., 1998).

2.4.3 Starch branching enzymes (SBEs, or Q-enzymes; EC 2.4.1.18)

Several isoforms of SBEs have been identified in developing storage tissues of higher plants (Boyer and Preiss, 1978). SBEs can be separated into two major groups. SBE type I (or class B) comprises SBEI from maize (Baba et al., 1991), wheat (Morell et al., 1997, Repellin et al., 1997, Båga et al., 1999b), potato (Kossman et al., 1991), rice (Kawasaki et al., 1993), and cassava (Salehuzzaman et al., 1992), and SBEII from pea (Burton et al., 1995). The other group, SBE type II (or class A), comprises SBEII from maize (Gao et al., 1997), wheat (Nair et al., 1997), potato (Larsson et al., 1996), and *Arabidopsis* (Fisher et al., 1996), SBEIII from rice (Mizuno et al., 1993), and SBEI from pea (Bhattacharyya et al., 1990). SBEI and SBEII are generally immunologically unrelated but have distinct catalytic activities. SBEI transfers long glucan chains and prefers amylose as a substrate, while SBEII acts primarily on amylopectin (Guan and Preiss, 1993). In endosperm of maize (Takeda et al., 1993), rice

(Yamanouchi and Nakamura, 1992), and barley (Sun et al., 1998), SBEII is further subclassified into SBEIIa and SBEIIb, each of which differs slightly in catalytic properties. The two SBEII forms are encoded by different genes and expressed in a tissue-specific manner (Gao et al., 1997, Fisher et al., 1996). Expression patterns of SBEIIa and SBEIIb in a particular tissue are specific to plant species. For example, the endosperm-specific SBEII in rice is SBEIIa (Yamanouchi and Nakamura, 1992), while that in barley is SBEIIb (Sun et al., 1998).

SBEs are involved mainly in amylopectin synthesis. This is recently supported by the production of high-amylose transgenic potato lines with reduced SBEI and II activities (Jobling et al., 1999, Schwall et al., 2000). Most SBEs characterized to date are in the 80- to 100-kDa molecular mass range. However, a novel SBEI of 152 kDa, namely SBEIc, was recently isolated from developing endosperm of wheat (Båga et al., 2000). The novel SBEIc is preferentially associated with A-type (large) starch granules from wheat endosperm and the immunologically related polypeptide of similar mass was exclusively observed in plant species exhibiting bimodal starch granule size distribution (Peng et al., 2000). Therefore, Peng et al. (2000) suggested that SBEIc might function in synthesis of amylopectin content in A-type starch granules of plants with bimodal starch granule size distribution.

2.5 Starch degrading enzymes

Cereal seeds store energy in the form of starch, which is degraded during germination to provide energy for growing seedlings before the onset of photosynthesis. Because intramolecular linkages of starch are composed of two different glucosidic bonds, hydrolysis of starch requires both α-1,4- and α-1,6-amylolytic enzymes. While some enzymes, for example amyloglucosidase, hydrolyse both types of linkages without discrimination, the others specifically hydrolyse only α-1,4 or α-1,6 linkages, for example amylases, and starch debranching enzymes, respectively. This review includes all the enzymes that can attack α-1,4 and α-1,6 linkages of starch, although some, for example amyloglucosidase and indirect debranching enzymes, do not exist in plants.

2.5.1 α-1,4-targeting enzymes

Amylolytic enzymes that attack α-1,4-glucosidic linkages in starch may function either as a hydrolase, or a transferase. The most important α-1,4-hydrolytic enzymes are the amylases, while phosphorylase and disproportionating enzyme are examples of starch degrading enzymes with transferase activity.

2.5.1.1 Amylases

According to the mode of action, amylases can be classified into two subgroups. Endoamylases are capable of random hydrolysis of internal α-1,4-glucosidic bonds regardless of the location of α-1,6 linkages. On the other hand, exoamylases can only hydrolyse linear α-glucan chains from the non-

reducing end and their hydrolytic action is terminated once the enzymes reach α-1,6 linkages.

All α-amylases (α-1,4-glucan-4-glucanohydrolase; EC 3.2.1.1), found in diverse groups of organisms including bacteria, fungi, plants and animals, have endo-action. Based on random hydrolytic activity of the enzyme, hydrolysis of starch with α-amylase produces a mixture of glucose, maltose, and oligosaccharides as the product, leaving smaller molecular weight branched glucan chains, α-limit dextrins, as the remaining substrate. The catalytic activity of α-amylases is Ca^{2+}-dependent. Various isoforms of α-amylases that are encoded by a multigene family have been identified in germinating cereal grains (Sutliff et al., 1991). Expression of different isoforms of α-amylases is observed in a tissue-specific manner (Karrer et al., 1991, Baulcombe et al., 1987) and is influenced by prolonged germination (O'Neill et al., 1990) and the presence of plant hormone gibberellic acid (Baulcombe and Buffard, 1983, Rogers, 1985).

Based on its exo-action, β-amylase (α-1,4-glucan maltohydrolase; EC 3.2.1.2) cleaves alternate glucosidic bonds of starch in a stepwise fashion starting at the non-reducing end releasing maltose as the product. The hydrolytic action of β-amylase is inhibited when α-1,6 branches are present, thus leaving the non-hydrolytic branched substrate called β-limit dextrin. Unlike α-amylase, the sources of β-amylases are restricted to plants and microorganisms (Pandey et al., 2000). In barley (Shewry et al., 1988), wheat (Daussant and Lauriere, 1990), and rye (Daussant et al., 1991), two forms of β-amylases, ubiquitous and endosperm-specific isoforms, have been identified.

The endosperm-specific β-amylase is synthesized during seed development and deposited in the endosperm in an enzymatically inactive or latent form (Hara-Nishimura et al., 1986). The C-terminal region of the latent β-amylase contains glycine-rich repeats that undergo proteolytic cleavage during germination (Yoshigi et al., 1994, Rorat et al., 1991). Cleavage of the C-terminal tail of barley β-amylase greatly enhances thermostability and starch binding ability of the enzyme and consequently converts the enzyme to an enzymatically active form (Ma et al., 2000). On the other hand, rice (Wang et al., 1996) and maize (Wang et al., 1997) β-amylases are synthesized *de novo* in aleurone layers during seed germination and do not contain the glycine-rich repeats at the C-terminus.

2.5.1.2 Starch phosphorylase (EC 2.4.1.1)

$$(\text{Glucose})_{n+1} + P_i \longleftrightarrow (\text{Glucose})_n + \alpha\text{-D-glucose-1-phosphate}$$

Phosphorylase is widely distributed among prokaryotic and eukaryotic organisms, namely starch or glycogen phosphorylase depending on the type of polysaccharide substrate. The enzyme catalyzes the reaction in which the α-1,4-linked glucose residue located at the non-reducing end of the glucan is removed as α-D-glucose-1-phosphate. Phosphorylase catalyzes both glucan-degrading and glucan-synthesizing activities. However, the main *in vivo* function of starch phosphorylase is generally assumed to be phosphorolytic glucan degradation (Steup, 1988). Phosphorylases from various organisms are composed of two identical subunits, each of which contains one covalently bound pyridoxal-5-phosphate (Newgard et al., 1989). Animal phosphorylase is

regulated by covalent modifications and/or allosteric effectors (Browner et al., 1992), while the regulatory mechanisms of plant phosphorylase have not yet been identified.

2.5.1.3 Disproportionating enzyme (D-enzyme, or 4-α-glucano transferase; EC 2.4.1.25)

$$Glucan_{(donor)} + Glucan_{(acceptor)} \longrightarrow Glucan_{(donor+acceptor-1)} + glucose$$

D-enzyme was first isolated from potato tuber (Peat et al., 1956). The enzyme transfers unbranched malto-oligosaccharide group from a donor α-1,4 glucan of at least three glucose residues (maltotriose) to a recipient oligosaccharide at the final expense of glucose formation. D-enzyme primarily catalyzes the condensation of short oligosaccharides to facilitate their degradation through phosphorylases or hydrolases (Lin and Preiss, 1988, Boos and Shuman, 1998). In addition, recent research has investigated the involvement of D-enzyme in the starch synthesis pathway. While Takaha et al. (1998) reported that a vast reduction of D-enzyme activity in antisense potato plants did not affect structure and amount of starch, Colleoni et al. (1999a) observed changes in structure of starch granule and amylopectin in *Chlamydomonas* mutants lacking D-enzyme activity. Therefore, Colleoni et al. (1999b) proposed that D-enzyme functioned directly in building the amylopectin structure, which is a basic requirement for normal starch granule formation.

2.5.2 α-1,6-targeting enzymes

Debranching enzymes exclusively hydrolyse α-1,6-glucosidic bonds in glucans that contain both α-1,4- and α-1,6-linked glucosyl moieties. Based on the mode of action, debranching enzymes are classified into two classes, direct and indirect debranching enzymes (Steup, 1988). Both direct and indirect debranching enzymes enhance accessibility of other hydrolytic enzymes to the branched substrates, resulting in complete degradation of the substrates to oligo- and monosaccharides.

2.5.2.1 Direct debranching enzymes

Direct debranching enzymes cleave α-1,6 glucosidic bonds of a branched α-glucan in one single step resulting in the liberation of the entire α-1,4-glucan chain. The enzymes are present in microorganisms and plants. Their physiological role is thought to primarily associate with starch degradation (Steup, 1988). Based on the substrate specificity, starch debranching enzymes have been further sub-classified into two types, limit dextrinase and isoamylase.

2.5.2.1.1 Limit dextrinase (R-enzyme, Pullulanase, α-dextrin 6-glucanohydrolase; EC 3.2.1.41)

Pullulanase was first isolated from *Klebsiella aerogenes* (formerly called *Aerobacter aerogenes*) (Bender and Wallenfels, 1961). The enzymes are widely distributed in bacteria (Nakamura et al., 1975, Hyun and Zeikus, 1985) and plants (Yellowlees, 1980, Yamada, 1980). The term pullulanase has been widely used in the literature to describe a starch debranching enzyme from plant species (Morinaga et al., 1997, Renz et al., 1998, Francisco et al., 1998,

Cho et al., 1999, and Beatty et al., 1999). Based on the catalytic activity, the plant enzymes are, however, inactive toward glycogen, while the bacterial enzymes have a significant, although incomplete, activity on glycogen (Kornacker and Pugsley, 1990). Therefore, Manners (1997) suggested that the name pullulanase should be restricted to enzymes of microbial origin while limit dextrinase is used for plants. In this study, the terms limit dextrinase and pullulanase are used based on the nomenclature suggested by Manners (1997).

Both limit dextrinase and pullulanase activities can be assayed using pullulan as the substrate. Pullulan, a polymer of α-1,6-linked maltotriose residues, is relatively resistant to other carbohydrate-degrading enzymes. Therefore, pullulan can be used to selectively determine limit dextrinase activity in crude plant extracts. Various methods to determine limit dextrinase activity have been reported. These include the measurement of reducing sugars released from hydrolysis of pullulan (Israilides et al., 1994) and the use of dye-crosslinked pullulan as the substrate (Serre and Laurière, 1990). After enzymatic hydrolysis of the dye conjugated-pullulan, the remaining substrate is separated by filtration or precipitation with ethanol. The color product remaining in the supernatant is proportional to the activity of limit dextrinase or pullulanase present in the sample. The dye-crosslinked pullulan can also be used in culture medium for the screening of bacterial colonies that produce and secrete pullulanase (Yang and Coleman, 1987), and in polyacrylamide gels for the detection of pullulan-degrading activity of a protein (Furegon et al., 1994).

Pullulanase or limit dextrinase activity in culture medium or polyacrylamide gels containing red pullulan can be observed by the presence of a clear area on the red background.

Besides pullulan, limit dextrinase specifically hydrolyses α-1,6-glucosidic bonds present in starch, amylopectin, and limit dextrin. The precise location of the α-1,6 linkages being hydrolyzed influences the ability of the enzyme to act on a specific substrate. Limit dextrinase requires the presence of two α-1,4-linkages adjacent to the α-1,6 linkage to be hydrolyzed (6^2-O-α-maltotriose) (Kennedy et al., 1987). Due to the importance of starch hydrolysis in the malting and brewing industry, limit dextrinase from barley grains has been studied extensively (Kristensen et al., 1998). Limit dextrinase in mature barley grains exists in bound or enzymatically inactive form which can be activated in the presence of reduced thiols (McCleary, 1992, Cho et al., 1999) or by proteolytic activation during germination (Longstaff and Bryce, 1993). In addition to the role of limit dextrinase in starch hydrolysis, the presence of limit dextrinase in developing storage tissues of several plant species has indicated the involvement of the enzyme in starch biosynthesis. In developing cereal grains, limit dextrinases have been reported in barley (Sissons et al., 1993), maize (Beatty et al., 1999), and rice (Nakamura et al., 1996b). The role of starch debranching enzymes in starch biosynthesis will be discussed.

2.5.2.1.2 Isoamylase (Glycogen 6-glucanohydrolase; EC 3.2.1.68)

Unlike limit dextrinase, isoamylase can not cleave α-1,6 linkages in molecules containing less than three α-1,4-linkages adjacent to the α-1,6 glucosidic bond to be hydrolyzed (6^3-α-maltosylmaltose) (Kennedy et al., 1987). Therefore, pullulan is not a substrate for isoamylase although it contains both α-1,4- and α-1,6-linkages. Isoamylase is enzymatically active toward highly branched polysaccharides, for example amylopectin and glycogen, while it shows low activity toward branched oligosaccharides. Due to the instability of the enzyme and unavailability of specific substrate, determination of isoamylase activity in plant extracts is very difficult. Therefore, early understanding of isoamylase was mainly obtained from microorganisms. The genes have been isolated and characterized from various bacterial species (Amemura et al., 1988, Krohn et al., 1997). Recently, the three-dimensional structure of *Pseudomonas* isoamylase was reported (Katsuya et al. 1998) and the essential residues of *Flavobacterium* isoamylase were identified using site-directed mutagenesis (Abe et al., 1999). Molecular understanding of isoamylases from plant origins has progressed since the isolation of a maize isoamylase cDNA (James et al., 1995). James et al. (1995) isolated a *sugary1* (*su1*) gene locus from developing maize kernels using transposon-tagging strategy. A cDNA copy of the *su1* transcript showed a high degree of deduced amino acid sequence identity to *Pseudomonas* isoamylase (Amemura et al., 1988). Biochemical assays confirm that the maize cDNA belongs to isoamylase-type

debranching enzyme (Rahman et al., 1998). Based on the information obtained from maize, isoamylase cDNA from rice (Fujita et al., 1999) and barley (Sun et al., 1999) have also been isolated.

To determine isoamylase activity, the enzyme must be first purified from other carbohydrate-degrading enzymes. Activity of purified isoamylase is generally determined using starch or amylopectin as the substrate. The activity of isoamylase is then calculated based on the number of reducing sugars released from the substrate. Alternatively, isoamylase activity can be qualitatively determined using starch- or amylopectin-containing polyacrylamide gel electrophoresis. After staining the gel with iodine, localization of a debranching enzyme can be observed as a blue band on a purple background, while amylase and starch branching enzyme give rise to a clear and red band on the purple background, respectively (Rammesmayer and Praznik, 1992). This technique can also be used to identify isoamylase-producing bacteria (Krohn et al., 1997). Since limit dextrinase can hydrolyse α-1,6-glucosidic bonds in starch and amylopectin, this technique can not be used to distinguish between isoamylase and limit dextrinase or pullulanase.

2.5.2.2 Indirect debranching enzymes

The action of indirect debranching enzymes is the cooperation of two enzyme activities, 4-α-glucanotransferase (EC 2.4.1.25) and amylo-1,6-glucosidase (EC 3.2.1.33). The glucanotransferase activity of the enzyme transfers three glucose residues from one short branch to the end of another, and the α-1,6-glucosidase activity hydrolyse the α-1,6 glucosidic bond that links

the remaining glucan chain to the main chain (Yang et al., 1992). It is believed that both transferase and glucosidase activities of the enzyme reside on a single polypeptide chain (White and Nelson, 1974). In combination with phosphorylase, the indirect debranching enzymes function in complete degradation of glycogen in mammals, yeast, fungi, and bacteria, thus are named glycogen debranching enzyme (Lee and Whelan, 1971). In humans, various tissue-specific isoforms of glycogen debranching enzymes have been identified, each of which is derived from a different transcript that occurs via alternative exon usage and/or different transcription start points of the existing gene (Bao et al., 1997).

2.5.3 α-1,4- and α-1,6-targeting enzymes

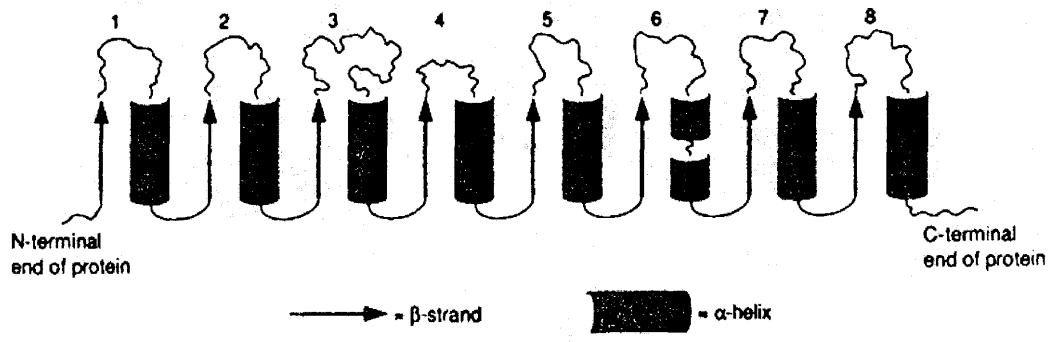
Amyloglucosidase (1,4-α-D-glucan glucohydrolase; EC 3.2.1.3) hydrolyses both α-1,4- and α-1,6-glucosidic bonds without discrimination. Amyloglucosidase is an exo-acting enzyme capable of hydrolysing the successive α-1,4-linkages between α-D-glucopyranosyl residues in starch and related polysaccharides with the production of β-D-glucose. Hydrolysis of the α-1,6-linkages occurs at a much slower rate as compared to that of α-1,4-glucosidic bonds. Amyloglucosidase is also known as glucoamylase, glucamylase, and γ-amylase. The enzyme is produced by microorganisms, almost exclusively by fungi genus *Aspergillus* , *Penicillium*, and *Rhizopus*.

2.6 Structural features of enzymes in the α-amylase family

X-ray crystallography of various α-amylases has shown that the polypeptide chains are made up of more than one folding unit or domain (Boel et al., 1990). According to the crystal structure of Taka-amylase A from *Aspergillus oryzae*, Matsuura et al. (1984) observed a catalytic $(\beta/\alpha)_8$-barrel structure. The $(\beta/\alpha)_8$-barrel domain consists of eight parallel β-strands surrounded by eight parallel α-helices, with an extra helix inserted after the sixth β-strand (Figure 2.5). The β-strands and helices alternate along the polypeptide chain and are linked together by irregular loops. Amino acid residues situated on the loops join the C-terminal end of each β-strand to the N-terminal end of the following helix, resulting in characteristic βα/βα/... connectivity. The $(\beta/\alpha)_8$-barrel structure has also been found in cyclodextrin glucanotransferase (EC 2.4.1.19) (Klein and Schulz, 1991) and enzymes that catalyze synthesis and/or hydrolysis of α-1,6-glucosidic linkages in starch and related polysaccharides, for example, branching and debranching enzymes of starch and glycogen (Jesperson et al., 1993). Recently, X-ray crystallography has confirmed the presence of $(\beta/\alpha)_8$-barrel supersecondary structure in the catalytic domain of *Pseudomonas* isoamylase (Katsuya et al., 1998). Although the overall structure of the $(\beta/\alpha)_8$-barrel domain appears similar in all the enzymes of the α-amylase family, small structural variations and different amino acid residues located within this region result in different substrate binding and catalytic activities of the enzymes (MacGregor, 1993).

Based on amino acid sequence comparison of α-amylases from bacteria, plants, and animals, four homologous regions likely to form the active and/or substrate-binding sites have been identified (Nakajima et al., 1986). Similar conserved regions have also been observed in other enzyme members of the α-amylase family (Table 2-2) (Janse et al., 1993 and Nakamura, 1996). It is shown that amino acid residues within the four homologous regions are highly conserved among the same type of enzyme regardless of organism sources (Nakamura, 1996). For example, amino acid sequences at the four conserved regions of plant isoamylases are closely related to those of bacterial isoamylases, but not to those of limit dextrinase from the same plant species. Therefore, amino acid substitutions within these regions can greatly affect catalytic activities of the enzymes. This has been confirmed by Kuriki et al. (1991), who demonstrated that substrate recognition and end product formation could be altered by changing amino acids within these regions. In addition, the location of conserved regions may also affect catalytic activity of the enzymes (Amemura et al., 1988). The conserved regions of α-1,4-degrading enzymes are mostly located near the N-termini, while those of α-1,6 hydrolytic enzymes are located apart from the N-termini. Therefore, catalytic activity of enzymes in α-amylase family is determined by characteristics of its conserved regions.

A.



B.

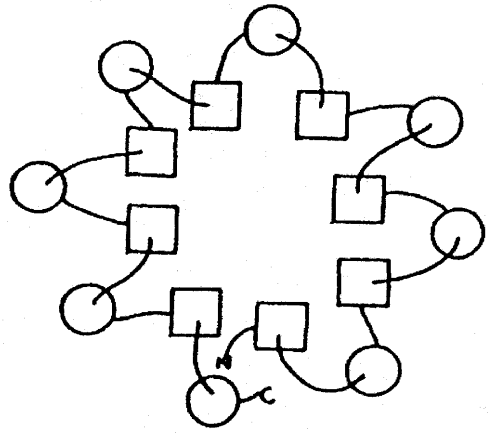


Figure 2.5 The $(\beta/\alpha)_8$-barrel domain of enzymes in the α-amylase family

The $(\beta/\alpha)_8$-barrel domain is shown in A in which loops 1 to 8 (wavy lines) link the C-terminal ends of β-strands to the N-terminal ends of adjacent helices (MacGregor, 1993). The secondary structure elements of the $(\beta/\alpha)_8$-barrel domain is shown in B, where the α-helices and β-strands are designated as circles and squares, respectively. The N and C indicate the N- and C-terminal ends of the polypeptide chain, respectively (Svensson, 1994).

Table 2-2 Conserved regions of enzymes in the α-amylase family

	I	*II*	*III*	*IV*	*Reference*
α-Amylase					
Aspergillus oryzae	DVVANH	GLRIDTVKH	EVLD	FVENHD	Matsuura et al., 1984
Porcine pancrease	DAVINH	GFRLDASKH	EVID	FVDNHD	Kluh, 1981
Branching Enzyme					
Type I (Class B)					
Rice endosperm	DVVHSH	GFRFDGVTS	EDVS	YAESHD	Mizuno et al., 1993
Maize endosperm	DVVHSH	GFRFDGVTS	EDVS	YAESHD	Fisher et al., 1993
Wheat endosperm	DVVHSH	GFRFDGVTS	EDVS	YAESHD	Morell et al., 1997
Pea embryo	DIVHSH	GFRFDGVTS	EDVS	YAESHD	Burton et al., 1995
Type II (Class A)					
Rice endosperm	DVVHSH	GFRFDGVTS	EDVS	YAESHD	Mizuno et al., 1992
Maize endosperm	DVVHSH	GFRFDGVTS	EDVS	YAESHD	Baba et al., 1991
Wheat endosperm	DVVHSH	GFRFDGVTS	EDVS	YAESHD	Nair et al., 1997
Pea embryo	DVIHSH	GFRFDGVTS	EDVS	YAESHD	Burton et al., 1995
Isoamylase					
Pseudomonas amyloderamosa	DVVYNH	GFRFDLASV	EWSV	FIDVHD	Amemura et al., 1988
Pseudomonas sp.	DVVYNH	GFRFDLASV	EWNG	FIDVHD	Tognoni et al., 1989
Flavobacterium sp.	DVVYNH	GFRFDLASV	EWNG	FVVAHD	Krohn et al., 1997
Rice endosperm	DVVFNH	GFRFDLASI	EAWD	FVCAHD	Fujita et al., 1999
Maize endosperm	DVVFNH	GFRFDLASI	EAWD	FVCAHD	James et al., 1995
Barley endosperm	DVVFNH	GFRFDLASI	EAWD	FVCAHD	Sun et al., 1999
Pullulanase					
Klebsiella aerogenes	DVVYNH	GFRFDLMGY	EGWD	YVSKHD	Katsuragi et al., 1987
Klebsiella pnemoniae	DVVYNH	GFRFDLMGY	EGWD	YVSKHD	Kornacker & Pugsley, 1990
Limit dextrinase					
Rice endosperm	DVVYNH	GFRFDLMGH	EGWD	YVSAHD	Nakamura et al., 1996a
Maize endosperm	DVVYNH	GFRFDLMGH	EGWN	YASAHD	Beatty et al., 1999
Barley endosperm	DVVYNH	GFRFDLMGH	EGWD	YVSAHD	Burton et al., 1999
Spinach leaf	DVVYNH	GFRFDLMGH	EGWD	YVSAHD	Renz et al., 1998
Glycogen debranching enzyme					
Human muscle	DVVYNH	GVRLDNCHS	ELFT	MDITHD	Yang et al., 1992
Rabbit muscle	DVVYNH	GVRLDNCHS	ELFT	MDITHD	Liu et al., 1993
Yeast	DIVFNH	GFRIDYCHS	ELSR	MDCTHD	Nakayama et al., 2001

2.7 Mutants lacking starch debranching enzyme activities

The maize mutants lacking a starch debranching enzyme activity are referred to as *sugary-1* (*su1*) (Pan and Nelson, 1984). Mature dried kernels of the homozygous *su1* mutant have a glassy, translucent, and shrunken appearance (Evensen and Boyer, 1986). Sumner and Somers (1944) reported that the storage polysaccharide in *su1* endosperms was not starch, but a highly branched, water-soluble polysaccharide called phytoglycogen. The level of phytoglycogen accumulated in *su1* endosperms can be up to 30% of the endosperm dry weight (Black et al., 1966). In addition to starch and phytoglycogen, the *su1* endosperms contain high concentration of sucrose and accumulate only small amount of amylopectin. Pan and Nelson (1984) observed significant reduction of limit dextrinase activity in *su1* kernels and the reduction of enzyme activity correlated with the *su1* gene dosage. Therefore, Pan and Nelson (1984) hypothesized that the *su1* mutation encoded the structural gene for limit dextrinase. Isolation and characterization of the gene on the *su1* locus of maize has, however, indicated that it codes for isoamylase (James et al., 1995, and Rahman et al., 1998). Immunoblot analysis using rice limit dextrinase antiserum detected an antigenically related protein in wild-type maize endosperm, but the protein was missing or dramatically reduced in *su1* endosperm (Rahman et al., 1998). Therefore, Rahman et al. (1998) suggested that *su1* function was necessary for accumulation of limit dextrinase in addition to the isoamylase for which it coded.

Mutants lacking a starch debranching enzyme activity have also been observed in rice (Matsuo et al., 1987). Nakamura et al. (1996b) observed that *sugary* mutants of rice were deficient in limit dextrinase activity. Similar to the pleiotropic effect of maize *su1* mutation, the reduction of limit dextrinase activity in *sugary* mutants of rice is not a primary effect coded by the *sugary* gene (Nakamura et al., 1996b). According to the chromosome mapping studies, the *sugary* locus of rice is located on chromosome 8 (Yano et al., 1984), while limit dextrinase gene is mapped on chromosome 4 (Nakamura et al., 1996a). Fujita et al. (1999) recently reported that the rice isoamylase gene is located on chromosome 8. However, it remains to be proven whether limit dextrinase gene is controlled by the isoamylase gene or another gene located on chromosome 8. Because isoamylase has no distinguishable specific substrate, isoamylase activity in *sugary* and non-*sugary* kernels of rice can not be directly determined. Although the rice isoamylase cDNA has been cloned, it remains to be tested whether the amounts of isoamylase transcript and protein could be observed in *sugary* endosperm of rice. Unlike in maize, it is not conclusive that the primary lesion of the rice *sugary* mutation is affected by the deficiency of isoamylase activity.

A *sugary*-like mutant (*sta7*) has also been observed in the unicellular alga *Chlamydomonas reinhardtii* (Mouille et al., 1996). The *Chlamydomonas sta7*-mutant accumulates glycogen-like material at the expense of starch. The *sta-7* mutant lacks a debranching enzyme activity, which has recently been identified as isoamylase (Dauvillée et al., 2000). Unlike *su1* mutant of maize

and *sugary* mutant of rice, limit dextrinase activity in *sta-7* mutant is not affected (Dauvillée et al., 2000). Therefore, the *sta-7* mutant has been used as a model to study the effect of isoamylase on structures of starch and starch granule. Consequently, the information obtained from *Chlamydomonas* could be used to explain the role of isoamylase in starch biosynthesis in higher plants.

2.8 Possible role of starch debranching enzymes in starch biosynthesis

Starch biosynthesis pathway is not completely understood due to the complicated structure of starch, highly ordered arrangement of amylose and amylopectin in starch granule, and participation of many enzymatic activities. While glycogen contains regularly spaced α-1,6 branches, the plant polysaccharide has asymmetric distribution of the α-1,6 branches in the unit amylopectin cluster. Since biosynthesis of both glycogen and starch requires pyrophosphorylase, synthase, and branching enzyme activities, this leads to the question of why catalytic activities of similar biosynthetic enzymes result in different end products. To test whether starch and glycogen branching enzymes had different branching activities, Guan et al. (1995) introduced maize starch branching enzyme into *E. coli* strain that lacks its own glycogen branching enzyme. Instead of producing amylopectin-like polysaccharide, the *E. coli* cells produced glycogen-like product. The result indicates that biosynthesis of amylopectin may require additional enzymatic reaction(s).

In contrast to amylose, mutants devoid of amylopectin have not yet been described in plants. Starch samples containing low amount of amylopectin

usually have deformed granule structure, while amylose-free starch granules show a normal growth ring pattern (Yamaguchi et al., 1979). Based on evidence obtained to date, it is believed that amylopectin is sufficient to generate full size granules by forming the network structure preventing the granules from collapsing. It is therefore suggested that major features of starch granule biogenesis might be explained if amylopectin biosynthesis is clearly understood. Since the *in vivo* synthesis of amylopectin requires an equilibrium between branching and debranching enzyme activities (Pan and Nelson, 1984), recent studies have focused on the action of starch debranching enzymes in starch synthesis rather than their hydrolytic function during germination. Two models have been proposed to explain the involvement of starch debranching enzymes, particularly isoamylase, in starch biosynthesis and starch granule formation.

2.8.1 Glucan-trimming model

Under specific growth conditions *Chlamydomonas* accumulates a polysaccharide that bears strong structural resemblance to maize endosperm storage starch (Delrue et al., 1992). In addition, mutations affecting starch synthetic enzyme activities in *Chlamydomonas* result in an identical fashion to those in maize (Ball et al., 1991). Therefore, it has been postulated that similar enzymes are responsible for starch synthesis in *Chlamydomonas* and in higher plants. In *sta-7* mutant, starch is completely replaced by phytoglycogen (Mouille et al., 1996) and limit dextrinase activity is not affected (Dauvillée, 2000). Therefore, Ball et al. (1996) suggested that debranching of starch with

isoamylase was a mandatory step in starch biogenesis in *Chlamydomonas*. In combination with information obtained from higher plants, a glucan-trimming model has been proposed to explain the involvement of isoamylase in amylopectin biogenesis (Figure 2.6) (Ball et al., 1996). The model also explains the highly ordered arrangement of amylopectin clusters and hence semi-crystalline structure of native starch granule (Myers et al., 2000).

According to the glucan-trimming model, starch synthases elongate α-1,4-linked glucan chains of amylopectin, which are located in crystalline lamellae of starch granule. Once the crystalline lamella has reached the critical size, random branches are introduced into amylopectin clusters by the *in vivo* excessive activities of branching enzymes. At this point, debranching enzyme activities will trim down the loosely branched glucans at the edge of growing amylopectin molecules. This step then yields the tightly spaced branches that will generate the next amorphous lamella of starch granule. In mutants lacking a starch debranching enzyme, the excessive activities of branching enzymes result in the formation of highly branched polysaccharides, leading to the production of phytoglycogen. Therefore, the mutants accumulate phytoglycogen at the expense of amylopectin.

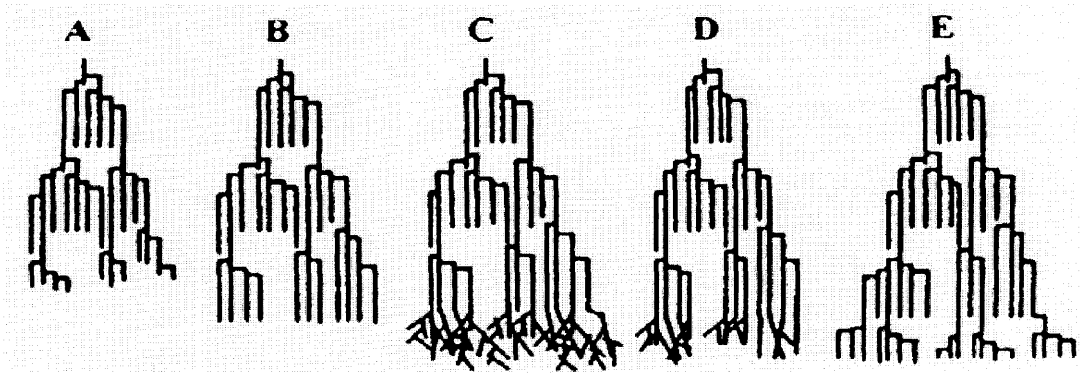


Figure 2.6 Glucan-trimming model
Elongation of glucan chains starts from a trimmed amorphous lamella depicted in A and proceeds through B until the critical size of crystalline lamella is reached. With the presence of high branching enzyme activities, random branches are introduced [C]. Debranching activities will simultaneously trim the loosely branched glucans [D]. This step prevents phytoglycogen synthesis and leaves out the tightly spaced branches that generate the next amorphous lamella [E] (Ball et al., 1996).

2.8.2 Soluble glucan-recycling model

Unlike the glucan-trimming model, the glucan-recycling model is mainly based on data obtained from transitory starch. Zeeman et al. (1998) suggested that leaf starch was a better system for study of starch biosynthesis pathway, because it is synthesized during a single photoperiod rather than days as in storage organs, thus, reduce the possibility of developmental changes in enzyme activities and isoform complements during granule formation. Similar to starch in storage organs, amylopectin molecule in transitory starch has a polymodal distribution of chain length (Matheson, 1996).

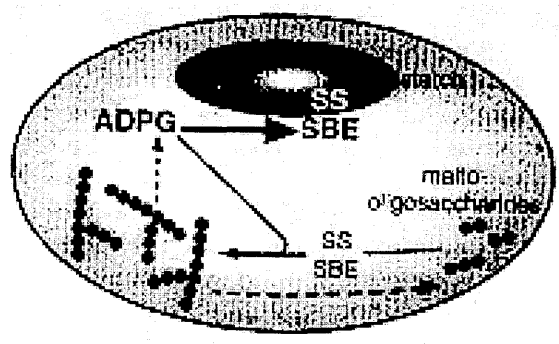
Based on the discovery of *Arabidopsis* mutants lacking a chloroplastic isoamylase, namely *dbe-1* (Zeeman et al., 1998), an alternative model to explain the involvement of isoamylase in amylopectin synthesis has been introduced (Smith, 1999). In *Arabidopsis dbe-1* mutants, the loss of chloroplastic isoamylase results in a vast reduction of starch and accumulation of phytoglycogen in *dbe-1* leaves. Both starch and phytoglycogen are accumulated simultaneously in the same chloroplasts of *dbe-1* mutant lines. Therefore, Zeeman et al. (1998) suggested that isoamylase would rather have an indirect role in determining amylopectin structure.

According to the glucan-recycling model (Figure 2.7) (Smith, 1999), starch synthases and starch branching enzymes have two functions *in vivo*. These include the synthesis of chloroplastic starch and soluble glucans. Only a small amount of soluble glucans is present in chloroplasts of wild type *Arabidopsis*. The presence of chloroplastic isoamylase prevents further growth

of the soluble branched glucans by recycling the glucans back to malto-oligosaccharides. In *dbe-1* mutants, the soluble branched glucans act as an alternative substrate for starch synthases and starch branching enzymes. These side chain reactions competitively withdraw starch synthases and starch branching enzymes from starch synthesis resulting in production of a small amount of starch and simultaneous accumulation of phytoglycogen in the same chloroplasts of *dbe-1* leaves.

As compared to the glucan-trimming model, the soluble glucan-recycling model has implied that phytoglycogen is not an intermediate of amylopectin synthesis but rather a separate soluble product made in stroma. Unlike the *Chlamydomonas sta-7* mutant, *sugary* mutants of maize and rice accumulate a small amount of starch in endosperm tissues (Pan and Nelson, 1984, Nakamura et al., 1996b). Therefore, Zeeman et al. (1998) and Smith (1999) suggested that the glucan-recycling model was suitable to explain the effect of *sugary* mutations in storage organ of maize and rice.

A.



B.

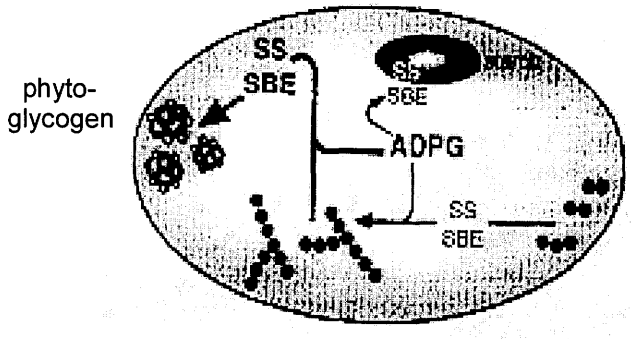


Figure 2.7 Soluble glucan-recycling model

A. In wild type *Arabidopsis* leaves, starch synthases (SS) and starch branching enzymes (SBE) synthesize starch from ADP-glucose (ADPG) and soluble glucans from malto-oligosaccharides. Starch debranching enzymes prevent the formation of phytoglycogen by recycling the soluble glucans to malto-oligosaccharides (Smith, 1999).

B. In *Arabidopsis dbe-1* leaves, glucan-recycling step does not occur. Therefore, side chain reactions of starch synthases and starch branching enzymes result in the formation of phytoglycogen from soluble glucans. Due to unavailability of starch synthases and starch branching enzymes, *dbe-1* leaves accumulate only small amount of starch (Smith, 1999).

3. MATERIALS AND METHODS

3.1 Plant material

Wheat genotype *Triticum aestivum* L. cultivar CDC Teal was grown in a greenhouse at 25 ± 2°C day and 20 ± 2°C night temperature under a 16 h photoperiod (93 $\mu E \cdot m^{-2} \cdot s^{-1}$) provided by banks of fluorescent tubes and incandescent bulbs. Floret tissues were collected before plants underwent anthesis. Spikes were tagged when the middle florets underwent anthesis. Developing kernels were collected at 5, 10, 15, and 20 days post-anthesis (dpa). The developing kernels used in enzyme assays were dissected under microscope to isolate embryo and only freshly isolated-endosperms were used in determination of enzyme activity. Leaf samples used in genomic DNA extraction were collected from 20-day-old plants, freeze-dried, and stored in a dessicator at room temperature.

To germinate wheat seeds *in vitro*, seeds were surface-sterilized by soaking in sodium hypochlorite for 30 min, then rinsed several times with sterile water. Seeds were germinated in a petri dish on a sterile filter paper soaked with sterile water. Germinating seed samples were collected after 1, 2, 3, 4, and 5 days of water imbibition. Roots and shoots were isolated from germinating seeds. All plant materials used in RNA extraction were rapidly frozen in liquid nitrogen and stored at -80°C.

3.2 Total starch assay

Concentration of starch in wheat kernels was determined using amyloglucosidase/α-amylase method as described by McCleary et al. (1994) (Total Starch, Megazyme International Ireland Inc.). Wheat kernels were milled, without subjected to a drying process, in a Udy cyclone mill with a 0.5-mm screen. A 100-mg milled sample was wet with 80% ethanol and treated by boiling for 6 min in 300 units thermostable α-amylase to partially hydrolyse the starch. The pH of the samples was adjusted by adding 4 ml of 200 mM sodium acetate buffer pH 4.5. Dextrins were quantitatively hydrolysed to glucose by incubation at 50°C for 30 min with 20 units amyloglucosidase at pH 4.5. The samples were adjusted to 100 ml total volume and centrifuged at 2,000 x g for 10 min. The amount of glucose in the supernatant was determined using glucose oxidase/peroxidase (GOPOD) reagent (Megazyme International Ireland Inc.). One litre of GOPOD reagent contains ≥12,000 units glucose oxidase, ≥650 units peroxidase, and 0.4 mmol 4-aminoantipurine in glucose reagent buffer (1 M potassium dihydrogen orthophosphate, 200 mM para-hydroxybenzoic acid). A 0.1-ml aliquot of the supernatant as described above was incubated with 3 ml GOPOD reagent at 50°C for 20 min. The absorbance at 510 nm of each sample was read against the reagent blank. The concentration of starch in a sample was calculated by comparing the amounts of glucose produced from the sample and from glucose standard (1 mg/ml). Concentration of starch was expressed as a percentage of fresh flour weight.

Starch % = $\Delta E \times F \times 1000 \times (1/1000) \times (100/W) \times (162/180)$

= $\Delta E \times (F/W) \times 90$

where ΔE = absorbance read against the reagent blank

F = conversion from absorbance of glucose standard to μg

= 100 μg of glucose/ absorbance for 100 μg of glucose

1000 = volume correction (0.1 ml taken from 100 ml)

1/1000 = conversion from μg to ml

100/W = factor to express starch as a percentage of flour weight

W = the weight in mg of the flour analysed

162/180 = adjustment from free glucose to anhydro-glucose which occurs in starch

3.3 Amylose/amylopectin assay

Amylose was determined using Con A precipitation method as described by Gibson et al. (1997) (Amylose/Amylopectin Assay Kit, Megazyme International Ireland Inc.). A 25-mg sample was solubilized by boiling for 15 min in 1 ml dimethyl sulfoxide (DMSO). Lipids were removed by precipitating the starch in 6 ml of 95% ethanol. The starch pellet was recovered by centrifugation at 2,000 x g for 5 min, then, redissolved by boiling for 15 min in 1 ml DMSO. The starch solution was diluted to 25 ml total volume with Con A solvent (180 mM sodium acetate, 900 mM NaCl, 0.9 mM $CaCl_2$, 0.9 mM $MgCl_2$, 0.9 mM $MnCl_2$ pH 6.4). Starch from a 0.5-ml aliquot was hydrolysed with 16.5 units amyloglucosidase and 2.5 units α-amylase. The sample was incubated with 4 ml GOPOD reagent at 40°C for 20 min, and the absorbance of the sample was

read at 510 nm against the reagent blank. Amylopectin in another aliquot (1.0 ml) was precipitated at room temperature for 1 h in the presence of 1.3 mg/ml Con A. After centrifugation at 15,000 x g for 10 min, amylose content in the supernatant was determined by treating the sample with amyloglucosidase/α-amylase solution as described above. The percentage of amylose was expressed as a proportion of total starch. The amount of amylopectin was indirectly determined by subtracting the amounts of amylose from total starch concentration.

$$\text{Amylose \%} = \frac{\text{Absorbance ConA supernatant}}{\text{Absorbance total starch aliquot}} \times \frac{153.8}{230} \times 100$$

$$= \frac{\text{Absorbance ConA supernatant}}{\text{Absorbance total starch aliquot}} \times 66.8$$

where 153.8 = dilution factor for ConA-treated sample

230 = dilution factor for total starch extract

3.4 Enzyme assays

3.4.1 Limit dextrinase

To extract proteins from developing wheat endosperm, the endosperm was ground at 4°C in extraction buffer (100 mM sodium maleate pH 5.0, 25 mM dithiothreitol [DTT]). The ground material was incubated on ice for 2 h to activate limit dextrinase (McCleary, 1992). Cell debris was removed by centrifugation at 15,000 x g for 2 x 30 min at 4°C. The supernatant was carefully decanted and kept on ice before use. Total protein concentration was determined using dye-binding method (Bradford, 1976). The samples were

diluted with 100 mM sodium maleate pH 5.0 (optimal pH for limit dextrinase from wheat) to obtain the required protein concentration of 0.5 μg/μl.

In this study, two limit dextrinase assays were performed. First, limit dextrinase activity was determined using azurine-crosslinked pullulan as the substrate (Limit DextriZyme, Megazyme International Ireland Inc.) (McCleary, 1992). A single use of the substrate (5 mg/ml) was in a tablet form. Crude extract of 500 μl was incubated at 40°C for exactly 20 min with a tablet of azurine-crosslinked pullulan. The reaction was terminated with the addition of 5 ml Trizma base (1%, Sigma). The remaining substrate was removed by filtration through a Whatman no.1 filter paper. The absorbance of the filtrate was read at 590 nm against the reaction blank. The reaction blank contained 500 μl of 100 mM sodium maleate buffer pH 5.0 instead of the plant extract and was processed in the same manner as the samples. Limit dextrinase activity was obtained from a standard curve calibrated with a purified standard sample of pullulanase from *Aerobacter aerogenes* (Megazyme International Ireland Inc.). One enzyme unit is defined as the amount of enzyme required to release one micromole of maltotriose reducing sugar equivalents per minute under the defined assay conditions.

The second limit dextrinase assay was performed using non-labeled pullulan as the substrate (Doehlert and Knutson, 1991). The assay contained 50 mM sodium citrate buffer pH 5.0, 5 mM DTT, 25 mg/ml pullulan, and 0.5μg/μl total protein concentration of plant extract. After incubation at 40°C for 20 min, a 100 μl aliquot was taken. The reaction was terminated by addition of 100 μl

dinitrosalicylic acid solution (40 mM 3,5-dinitrosalicylic acid, 0.4 N NaOH, 1 M sodium potassium tartrate). The sample was boiled for 5 min, and then diluted to 1 ml with water. Reducing value was determined at 520 nm as described by Bernfeld (1951). The enzyme activity was calculated from a maltotriose standard curve.

3.4.2 Amylolytic enzymes

In plant extracts, activity of isoamylase could not be accurately determined due to unavailability of a specific substrate. In this study, total amylolytic activity in developing wheat endosperm was determined using amylopectin as the substrate. The activity of isoamylase in developing wheat kernels was estimated by subtracting the total amylolytic activity with limit dextrinase activity.

Total amylolytic activity was determined by measuring the increase in blue value of amylopectin-iodine complex (Doehlert and Knutson, 1991). The reaction mixture contained 50 mM Hepes-NaOH pH 7.0, 5 mM DTT, and 100 μg amylopectin. After incubation at 30^0C for 30 minutes, 900 μL of color reagent (0.01 M I_2 and 0.5 M KI in H_2O) was added to the 100-μl reaction mixture. Increase in absorbance of amylopectin-iodine complex was detected from 500 to 700 nm, but the maximal increase generally occurred at 550 nm. Therefore, total amylolytic activity was determined by monitoring the changes in absorbance of iodine-amylopectin complex at 550 nm, and then calculated from a standard curve calibrated with dextrin (Sigma).

All enzyme assays were performed in a range at which the reaction velocity was proportional to the protein concentration and the incubation time. Each result is the mean ± S.D. of at least three replicate incubations.

3.5 Amplification of an isoamylase DNA fragment

A partial wheat isoamylase DNA fragment was amplified from a cDNA library (Nair et al., 1997) using polymerase chain reaction (PCR). To make phage DNA available for PCR amplification, the λ-ZAP vectors carrying wheat cDNA fragments were treated with 0.5% Tween 20, 1 mg/ml protease K in 1x PCR buffer (10 mM Tris [tris(hydroxymethyl) aminomethane]-HCl, 15 mM $MgCl_2$ pH 8.3). After incubation at 65°C for 45 min and 95°C for 10 min, 5 μl of the lysate was used in subsequent PCR reactions. Two oligonucleotide primers were synthesized based on the first (forward primer; 5′-GGGATGTTGTCTTCAATCATAC-3") and fourth (reverse primer; 5′-GTCCATCGTGTGCACATACAA-3′) conserved regions of maize isoamylase cDNA (James et al., 1995). The PCR reaction contained 0.2 mM dNTPs, 0.4 μM of each primer, 1.5 units *Taq* polymerase in 1x PCR buffer. The PCR was performed as follows: 94°C 45 s, 58°C 1 min, 72°C 2 min for 35 cycles, and additional annealing at 72°C for 15 min. The PCR products were visualized on 1% agarose gel electrophoresis in TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM ethylenediaminetetraacetic acid [EDTA] pH 8.0).

3.6 Cloning of PCR products

The PCR products were ligated to pCR2.1 vector (Original TA Cloning®, Invitrogen) (Figure 3.1). The ligation reaction contained 1 μl PCR product, 50 ng

pCR 2.1 vector, and 4.0 Weiss units T4 DNA ligase in 1x ligation buffer (6 mM Tris-HCl pH 7.5, 6 mM $MgCl_2$, 5 mM NaCl, 0.1 mg/ml bovine serum albumin, 7 mM β-mercaptoethanol, 0.1 mM ATP, 2 mM DTT, 1 mM spermidine). The ligation was performed overnight at 14°C. The ligated products were used to transform 50 μl INVαF′ *E.coli* competent cells (Invitrogen). First, a vial of competent cells was thawed on ice, and then one μmol of β-mercaptothanol and two μl of ligation reaction were added to the thawed cells. The ligation mixture was gently combined using a pipette tip, and then incubated on ice for 30 min. The sample was incubated at 42°C for exactly 30 s, and then transferred to an ice bath for 2 min. After addition of 250 μl SOC medium, the sample was incubated in a rotary-shaking incubator at 37°C for 1 h at 225 rpm. Aliquots of 50 μl and 200 μl from each transformation vial were spread on Luria-Bertani (LB) agar plates containing 50 μg/ml kanamycin and 1.6 mg X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) spread on the agar surface. After the liquid was absorbed, the plates were incubated at 37°C for at least 18 h. Plates were then shifted to 4°C for 2-3 h to allow the proper color development. Transformed colonies were selected based on blue-white appearance. White colonies were inoculated in LB broth containing 50 μg/ml kanamycin. The cultures were grown overnight at 37°C in a shaking incubator. To confirm the size of the insert, plasmids were isolated, and then subjected to EcoRI restriction digest.

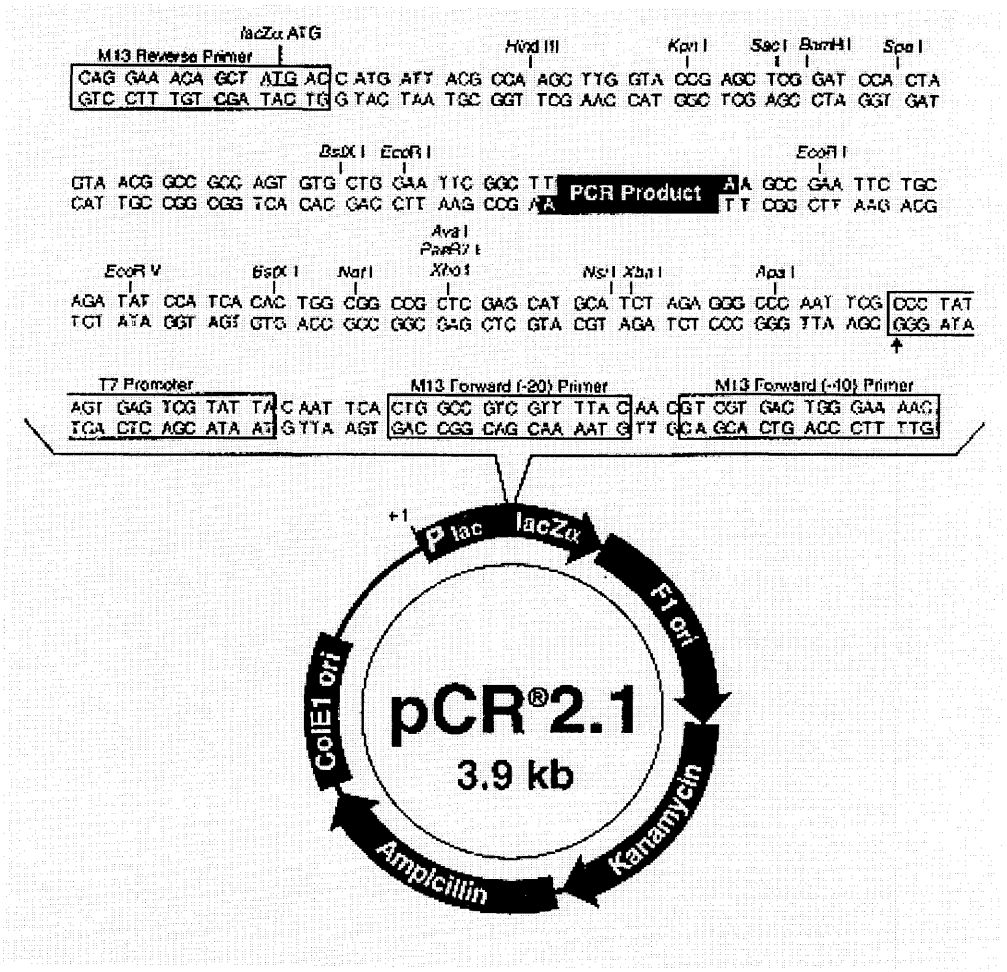


Figure 3.1 Vector map of pCR 2.1

3.7 Purification of plasmids

3.7.1 Quick method

Plasmids were isolated from bacterial cells using cetyl-trimethylammonium bromide (CTAB) method (Sal et al., 1989). The cationic detergent CTAB forms precipitate with nucleic acid while proteins and polysaccharides remain in the solution. The detergent is subsequently removed by resuspending the precipitate in NaCl followed by ethanol precipitation of nucleic acid. To isolate the plasmids, bacterial cells in a 1.5-ml culture was recovered by centrifugation at 15,000 x g for 2 min. The cell pellet was resuspended in 200 μl STET (8% sucrose, 50 mM Tris-HCl pH 8.0, 50 mM EDTA, 0.1% v/v Triton X-100). The cells were lysed by boiling for exactly 45 s in the presence of 1 μg/μl lysozyme. The sample was centrifuged at 15,000 x g for 10 min and the pellet was removed. The supernatant was treated with RNase A (250 μg/ml) at 68°C for 10 min. The DNA was precipitated in the presence of 15 μl CTAB solution (5% w/v). The pellet was recovered by centrifugation at 15,000 x g for 5 min, and then resuspended in 300 μl of 1.2 M NaCl. The DNA was precipitated with 750 μl ethanol followed by centrifugation at 15,000 x g for 5 min. The DNA pellet was washed in 80% ethanol, centrifuged at 15,000 x g for 5 min, and dissolved in 20 μl TE buffer (10 mM Tris pH 8.0, 1 mM EDTA).

3.7.2 Ultrapure method

The plasmids used in nucleotide sequencing were purified using an anion exchange resin (QIAGEN), which results in the isolation of ultrapure supercoiled plasmid DNA. The purification protocols as provided by the

manufacturer are based on an alkaline lysis, followed by binding of plasmid DNA to the resin under appropriate low salt and pH conditions. RNA, proteins, dyes, and low molecular weight impurities are removed by a medium salt wash. The DNA is then eluted with a high salt buffer, subsequently concentrated and desalted by isopropanol precipitation.

Bacterial culture grown in 2-ml LB medium containing appropriate amount of antibiotic (50 μg/ml ampicillin for *E. coli* INVαF′ carrying pCR2.1 vector and SolR cells carrying pBluescript vector or 30 μg/ml kanamycin for *E. coli* BL21(DE3) carrying pET-28(a) vector) was centrifuged at 15,000 x g for 5 min. The bacterial pellet was resuspended in 0.3 ml of Buffer P1 (50 mM Tris-HCl pH 8.0, 10 mM EDTA, 100 μg/ml RNase A). The cells were incubated at room temperature for 5 min with 0.3 ml of Buffer P2 (200 mM NaOH, 1%SDS). After addition of 0.3 ml of chilled Buffer P3 (3 M potassium acetate pH 5.5), the sample was incubated on ice for 5 min followed by centrifugation at 15,000 x g for 10 min at 4°C. The supernatant was applied to a QIAGEN-tip 20, which was equilibrated with 1 ml Buffer QBT (750 mM NaCl, 50 mM MOPS [3-(N-morpholino)propanesulfonic acid] pH 7.0, 15% isopropanol, 0.15% Triton X-100). The QIAGEN-tip 20 was washed with 4 x 1 ml Buffer QC (1 M NaCl, 50 mM MOPS pH 7.0, 15% isopropanol). The plasmid DNA was then eluted with 0.8 ml Buffer QF (1.25 M NaCl, 50 mM Tris-HCl pH 8.5, 15% isopropanol). The DNA was precipitated with 0.7 vol isopropanol. The pellet was recovered by centrifugation at 15,000 x g for 30 min, washed in 1 ml of 70% ethanol, air-dried, and dissolved in 20 μl TE buffer pH 8.0.

3.8 Labeling of isoamylase DNA probes

3.8.1 Radiolabeling method

The pCR2.1 vectors carrying a 619-bp wheat isoamylase DNA fragments were digested with EcoRI. The EcoRI-treated DNA fragments of wheat isoamylase were purified from an agarose gel using QIAGEN gel extraction kit (QIAGEN). The purified DNA was randomly labeled with [^{32}P]-dCTP using Rediprime DNA labeling systerm (Amersham). The DNA was diluted to a concentration of 20 ng in 45 μl of sterile water, denatured by heating to 95-100°C for 5 min, and then snap-cooled on ice for 5 min. The denatured DNA was mixed with Rediprime labeling mix (Amersham) and 50 μCi Redivue [^{32}P]-dCTP. The labeling reaction was incubated at 37°C for at least 30 min, and then terminated by adding 5 μl of 0.2 M EDTA. The radiolabeled probe was purified using NAP™ 5 Columns (Amersham). Before adding to the hybridization buffer, the labeled DNA was denatured by heating to 95-100°C for 5 min, and then chilled on ice.

3.8.2 Digoxigenin (DIG)-labeling method

DIG-dUTP was randomly incorporated into a partial isoamylase DNA fragment during the PCR (PCR DIG probe systhesis, Boehringer Mannheim). The 50-μl PCR reaction contained 0.2 mM of each dATP, dCTP, and dGTP, 0.13 mM dTTP, 0.07 mM DIG-11-dUTP, 0.4 μM of each primer, 2.6 units Expand™ High Fidelity polymerase (Boehringer Mannheim), and 0.1 ng DNA template in 1x PCR buffer. The pCR2.1 vector carrying a 619-bp wheat isoamylase DNA fragment was used as the template in PCR reactions. The

PCR was performed as follows: 94°C 45 s, 58°C 1 min, 72°C 2 min for 35 cycles, and additional annealing at 72°C for 15 min. As compared to the unlabeled control, a higher molecular weight shift of the DIG-labeled PCR product was detected by agarose gel electrophoresis.

A full-length isoamylase cDNA was labeled with DIG-dUTP using random primed DNA labeling method (DIG High Prime, Boehringer Mannheim). The 2.6-kb cDNA fragment was excised from the pBluescript vector at NotI and XhoI sites. The 2.6-kb wheat isoamylase cDNA was diluted to a concentration of 1 μg in 16 μg of sterile water, denatured by boiling for 10 min, and then snap-cooled on ice for 5 min. The labeling reaction (20 μl) contained 1 μg denatured DNA, 4 units Klenow enzyme, 4 nmol of each dATP, dCTP, and dGTP, 2.6 nmol dTTP, and 1.4 nmol DIG-11-dUTP. After overnight incubation at 37°C, the labeling reaction was terminated by adding 2 μl of 0.2 M EDTA pH 8.0. Before adding to the hybridization buffer, the DIG-labeled probe was denatured by heating to 100°C for 10 min, and then chilled on ice.

3.9 Screening of a wheat cDNA library

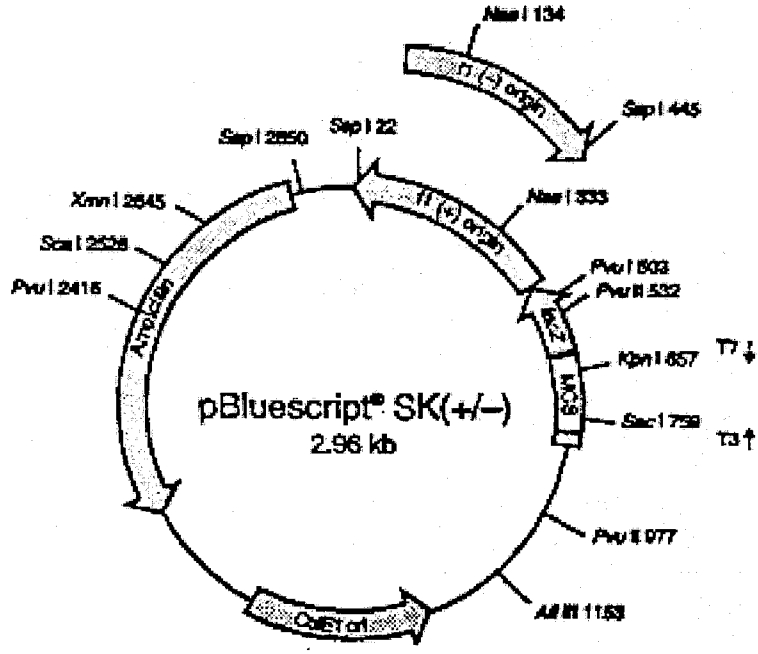
A wheat cDNA library was constructed in λ-ZAP vector using poly (A)+ RNA from 12 dpa wheat kernels (*Triticum aestivum* L. cv. Fielder) (Nair et al., 1997). Wheat cDNA fragments were ligated to the Uni-ZAP XR vector arms via EcoRI and XhoI sites. To plate the Uni-ZAP XR phage particles, XL1-Blue MRF′ host strain was grown in LB broth containing 0.2% (v/v) maltose and 10 mM $MgSO_4$. The XL1-Blue MRF′ culture was diluted to A_{600} = 0.5 with 10 mM $MgSO_4$. An aliquot of the library suspension containing ~30,000 plaque-forming

units (pfu) was mixed with 600 μl A_{600} = 0.5 XL1-Blue MRF′ cells. The phage-bacterial mixture was incubated at 37°C for 15 min. Ten ml of 50°C prewarmed NZY top agar was added to the phage-bacterial mixture. The mixture was spread evenly onto a 150-mm NZY agar plate. The plate was incubated at 37°C for 8 h and plaques were replica plated onto a Hybond-N+ nylon membrane (Amersham). The membrane was treated for 7 min in denaturation solution (1.5 M NaCl, 0.5 M NaOH), 2 x 3 min in neutralization solution (1.5 M NaCl, 0.5 M Tris-HCl pH 7.2, 1 mM EDTA), and then washed briefly in 2x SSC (0.3 M NaCl, 30 mM sodium citrate pH 7.0). The membrane was air-dried and UV-crosslinked with 1.2 x 10^5 μJ of UV energy (Stratalinker, Stratagene). Approximately 1 x 10^6 pfu were screened using the 619-bp wheat isoamylase DNA fragment, labeled with [^{32}P]-dCTP, as a probe. To identify phage particles carrying a wheat isoamylase cDNA fragment, the membrane was incubated overnight at 65°C in hybridization buffer (0.5 M Na_2HPO_4 pH 7.2, 7% SDS) containing 0.8 ng/ml ^{32}P-labeled probe. The membrane was washed at 65°C for 30 min each in 5% SEN buffer (40 mM Na_2HPO_4 pH 7.2, 5% SDS, 1 mM EDTA) and 1% SEN buffer (40 mM Na_2HPO_4 pH 7.2, 1% SDS, 1mM EDTA). The membrane was exposed to an X-ray film for a period of time depending on signal intensity. The film was aligned to the original plate and the media exposing a positive signal was cut and put into 1 ml SM buffer (0.1 M NaCl, 15 mM $MgSO_4$, 50 mM Tris-HCl pH 7.5, 0.01% gelatin) containing 20 μl chloroform. Secondary and/or tertiary screening were performed until a single plaque was obtained. The plaque was cut from agar plate and transferred to a

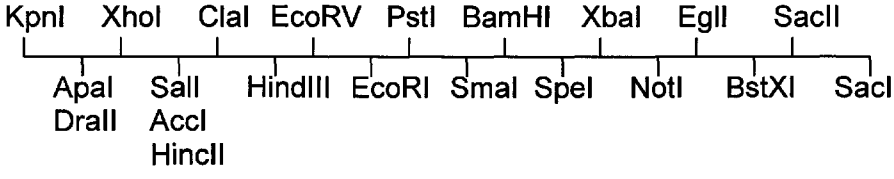
sterile tube containing 500 μl SM buffer and 20 μl chloroform. This phage stock is stable for up to 1 year at 4°C.

3.10 *In vivo* excision of the Bluescript phagemid

The Bluescript phagemid was excised from the Uni-ZAP XR vector using the ExAssist/SOLR system (Stratagene, La Jolla, CA). First, XL1-Blue MRF′ cells were grown in LB broth to mid-log phase (A_{600} = 0.2-0.5). The cells were pelleted (1,500 x g) and resuspended at A_{600} = 1.0. A mixture of 200 μl of A_{600} = 1.0 XL1-Blue MRF′ cells, 250 μl of a single-clone phage stock, and 1 μl of ExAssist helper phage was incubated at 37°C for 15 min. After addition of 3 ml LB broth, the phage mixture was further incubated with shaking (200 rpm) at 37°C for 2-2.5 h. The sample was centrifuged at 2,000 x g for 15 min. The supernatant was transferred to a fresh tube, heated at 70°C for 15 min, and then centrifuged at 4,000 x g for 15 min. The supernatant, which contained the excised Bluescript phagemids packaged as filamentous phage particles, was transferred to a sterile tube and stored at 4°C until ready to use. To plate the excised phagemids, 10 μl of the excised Bluescript phage solution was incubated with 200 μl freshly grown SOLR cells (A_{600} = 1.0) at 37°C for 15 min. A 50-μl aliquot was plated on a LB plate containing 50 μg/ml ampicillin. The plate was incubated overnight at 37°C. At this point, the pBluescript (Figure 3.2) carrying a fragment of wheat cDNA could be manipulated as a plasmid.



Multiple cloning site (MCS)



The SacI site lies immediately downstream from the bacteriophage T3 promoter and the KpnI site lies immediately downstream from the bacteriophage T7 promoter.

Figure 3.2 Vector map of pBluescript SK (+/-)

3.11 Expression of wheat isoamylase in *E.coli*

3.11.1 Isoamylase expression construct

Wheat cDNA fragments were inserted to the pBluescript at the EcoRI and XhoI sites. The entire 2.6-kb wheat isoamylase cDNA was excised from the pBluescript at the NotI (located 37 bp upstream of the EcoRI site) and XhoI sites located in the polycloning site of the vector. The 2.6-kb wheat isoamylase DNA fragment and the 3.0-kb fragment of pBluescript were fractionated on 1% agarose gel. The 2.6-kb fragment was purified from the gel using QIAGEN Gel Extraction Kit (QIAGEN). The resulting wheat cDNA fragment (2,640 bp) was ligated to NotI-XhoI treated pET-28(a) vector (Novagen, Figure 3.3). The 10-μl ligation reaction contained 1:3 molar ratio of vector DNA to insert DNA, 1 unit T4 DNA ligase (Boehringer Mannheim) in 1x T4 ligase buffer (66 mM Tris-HCl, 5 mM $MgCl_2$, 1 mM DTT, 1 mM ATP pH 7.5). The ligation was performed at 4°C for 16 h. The expression construct was used to transform freshly prepared competent cells of *E.coli* BL21(DE3) (Novagen) using standard transformation method (Sambrook et al., 1989). Transformed cells were grown on LB plates containing 30 μg/ml kanamycin. Several colonies were selected and further analysed to determine whether the plasmids carried the 2.6-kb wheat isoamylase cDNA fragment in the correct reading frame.

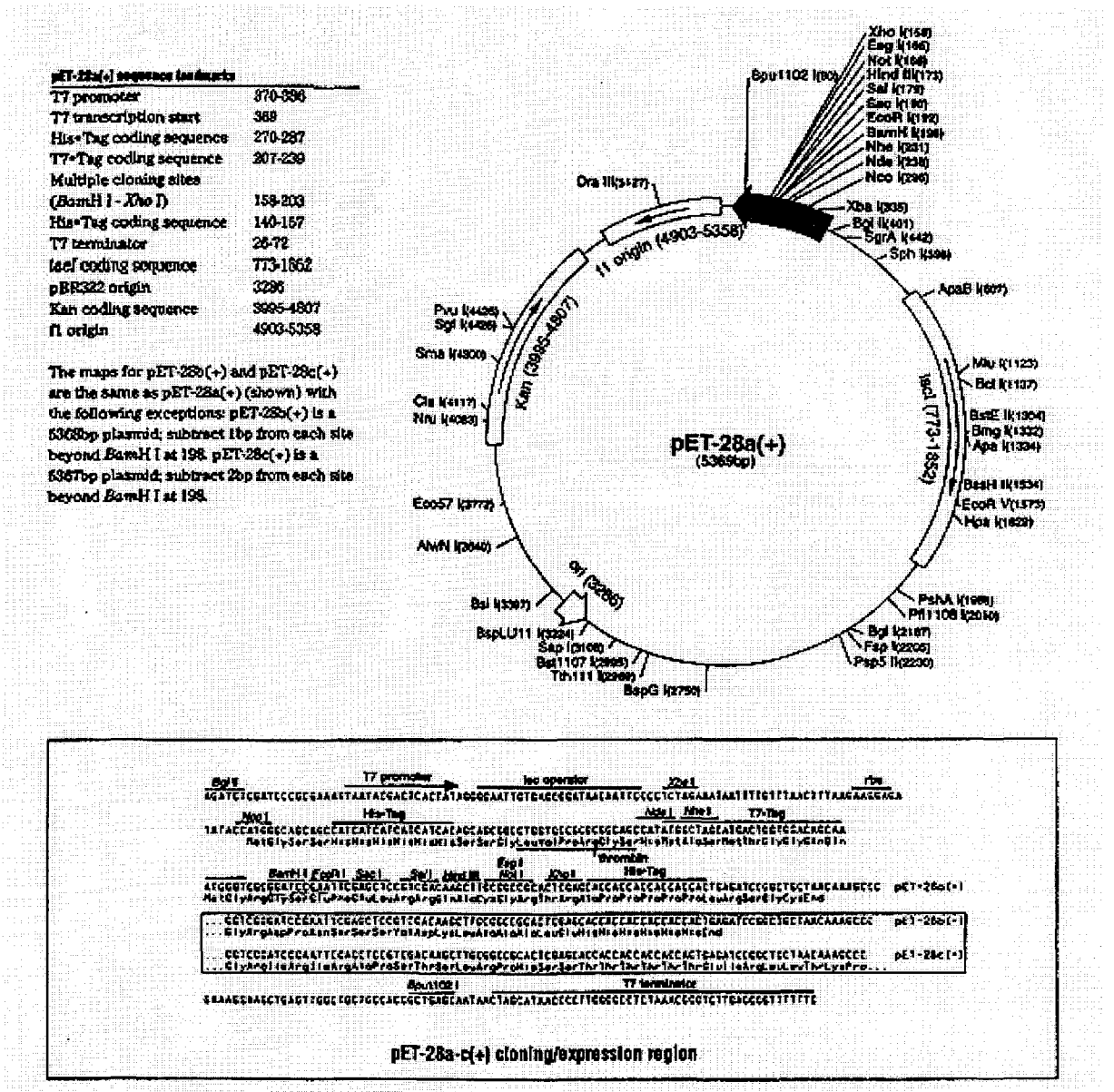


Figure 3.3 Vector map of pET-28(a-c)

The pET-28a-c(+) vectors carry an N-terminal His·Tag/thrombin/T_7·Tag configuration plus an optional C-terminal His·Tag sequence. The sequence on the circle map is numbered by the pBR322 convention, so the T_7 expression region is reversed on the circular map. The cloning/expression region of the coding strand transcribed by T_7 RNA polymerase is shown in box.

3.11.2 Preparation of fresh competent cells

Competent cells to be transformed with the isoamylase expression construct were freshly prepared by treatment of bacterial cells with $CaCl_2$ (Sambrook et al., 1989). The glycerol-frozen cells of *E.coli* BL21(DE3) (Novagen) were grown on SOB plates at 37°C for 16 h. A single colony was transferred into 100 ml of SOB medium in a 1-litre flask. The culture was grown at 37°C with vigorous shaking (300 rpm) until the A_{600} reaches 0.6. The cells were recovered by centrifugation at 4,000 x g for 10 min at 4°C, and the media was carefully removed. The cells were resuspended in 10 ml of ice-cold 0.1 M $CaCl_2$ and incubated on ice for 10 min. The cell pellet was recovered, and then resuspended in 2 ml of ice-cold 0.1 M $CaCl_2$ for each 50 ml of original culture. The fresh competent cells were ready to use. To store the competent cells, aliquots of the suspensions were quickly dispersed into chilled, sterile microfuge tubes, immediately frozen by immersing the tubes in liquid nitrogen, and stored at -70°C.

3.11.3 Induction of recombinant protein expression

The *E.coli* BL21(DE3) that carried the 2.6-kb wheat isoamylase cDNA in the correct reading frame was chosen for the expression study of recombinant isoamylase. Expression of the recombinant protein in bacterial culture grown at mid-log phase was induced by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Uninduced cells were collected and used as a negative control. The induced cells were harvested after 4 h of IPTG induction. The cells were collected by centrifugation at 5,000 x g at 4°C for 5 min,

resuspended in 1/4 culture volume of cold 50 mM Tris-HCl pH 8.0, 2 mM EDTA buffer, and centrifuged as above. The pellets were stored at -20°C until use.

To extract cellular proteins, the harvested BL21(DE3) cells were resuspended in 1/10 culture volume of 50 mM Tris-HCl pH 8.0, 2 mM EDTA, and then incubated at 30°C for 15 min with 100 μg/ml lysozyme and 0.1% (v/v) Triton X-100. The cells were lysed at 4°C by sonication at 10 s pulses at a high output setting. Proteins localized in soluble and insoluble fractions of bacterial cells were separated by centrifugation at 12,000 x g for 15 min at 4°C, and then subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

3.12 Polyclonal antibody preparation

To limit bacterial protein contamination, proteins extracted from insoluble fraction of IPTG-induced *E. coli* BL21(DE3) cells were fractionated on a SDS-PAGE gel. The *E. coli* BL21(DE3) cells that carried wheat isoamylase expression construct were grown in LB broth containing 30 μg/ml kanamycin. After 4 h of IPTG induction, 2 ml of the culture were collected and proteins from insoluble fraction of the cells were extracted and fractionated on a 20 x 16 x 0.75 mm SDS-PAGE gel. The gel was stained for 30 min in Coomassie Blue R (0.25% in water), and then destained in water until the protein bands were visible. The protein band with the size corresponding to the recombinant wheat isoamylase was excised from a polyacrylamide gel and freeze-dried. The dried gel was ground into fine powder and mixed with 1 ml of Freund's adjuvant, and then injected to cereal-starved rabbits. The rabbits were immunized three times

at two-week intervals with similar amount of protein. The serum was collected two weeks after the last injection, pooled, and used as a source of polyclonal antibodies. The rabbit antibodies were used at 1:5,000 dilution in all Western blot analyses.

3.13 SDS-PAGE

Proteins were fractionated on a 20 x 16 x 0.75 mm polyacrylamide gel according to standard method described by Laemmli (1970). The separating gel contained 8% acrylamide (29:1, acrylamide: bisacrylamide), 375 mM Tris pH 8.8, 0.1% SDS, and 0.1% ammonium persulfate. The stacking gel contained 5% acrylamide (29:1, acryl:bis), 125 mM Tris pH 6.8, 0.1% SDS, and 0.1% ammonium persulfate. The gel was run in Bio-Rad Protean II gel appparatus at a constant current (8 mA/gel) at 4°C for 12 h in Tris-glycine electrophoretic buffer (25 mM Tris, 250 mM glycine pH 8.3, 0.1% SDS). Protein bands were visualized by subjecting the gel to Coomassie Blue R-250 or silver staining. Alternatively, a specific protein could be detected via antigen-antibody interaction in immunoblot analysis.

3.14 Immunoblot analysis

Proteins fractionated on the SDS-PAGE gel were transferred to a polyvinylidene difluoride (PVDF) membrane according to a standard method described by Towbin et al. (1979). Electrophoretic transfer of the proteins to Immobilon™-P membranes (Millipore) was performed in Bio-Rad Trans-Blot cell at 50 volts for 8 h at 4°C in transfer buffer (25 mM Tris, 192 mM glycine pH 8.3, 20% methanol). The non-specific signals were blocked by incubating the

menbrane in TBST buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Tween 20) containing 3% bovine serum albumin for at least one hour. The membrane was then incubated with TBST buffer containing suitable amount of the primary antibody. The primary antibodies used in this study were either alkaline phosphatase-conjugated T_7·tag antibody (1:10,000 dilution, Novagen) or rabbit anti-wheat isoamylase antibody (1:5,000 dilution). After incubation for 30 min with T_7·tag antibody or 4 h with rabbit antibody, the membrane was washed for 3 x 10 min in TBST buffer. The membrane exposed to T_7·tag antibody was then subjected to signal detection in the presence of chromogenic substrates (nitro blue tetrazolium; NBT, and 5-bromo-4-chloro-3-indoyl phosphate; BCIP, Stratagene). For immunoblot analysis using the rabbit antibody, positive signals were detected using secondary antibody conjugated with alkaline phosphatase. The membrane was incubated for 1 h in TBST containing alkaline phosphatase conjugated goat anti-rabbit antibody (1:5,000 dilution, Stratagene). The membrane was washed as described above and then subjected to chromogenic signal detection (338 μg/ml NBT and 175 μg/ml BCIP in 100 mM Tris-HCl pH 9.5, 100 mM NaCl, 1 mM $MgCl_2$). Once the color reached desired intensity, the reaction was terminated by rinsing the membrane in 20 mM Tris-HCl pH 8.0, 5 mM EDTA buffer.

3.15 Zymogram detection of starch debranching enzymes

Starch debranching enzyme activity can be qualitatively determined on starch-containing polyacrylamide gel (Mouille et al., 1996). Recombinant proteins (300 μg) extracted from bacterial cells were fractionated on a SDS-

PAGE gel (20 x 16 x 1.5 mm) containing 0.3% soluble potato starch (Sigma). Electrophoresis was performed at a constant current (10 mA/gel) at 4°C for 15 h in 25 mM Tris-glycine pH 8.3, 1 mM DTT, 0.1% SDS buffer. After electrophoresis, the gel was incubated for 1 h with gentle shaking in 40 mM Trizma base (Sigma) at room temperature to remove SDS. Proteins were renatured by overnight incubation in 25 mM Tris-glycine pH 8.3, 20 mM DTT at room temperature. The gel was rinsed with water before staining with 0.25% KI, 0.025% I_2 solution. Starch debranching enzymes could be visualized as a blue band on a purple background. To determine the ability to use pullulan as the substrate, polypeptides from a starch-containing gel were electroblotted onto a gel containing 1% (w/v) red pullulan (Steup and Garbling, 1983, Zeeman et al., 1998). The polypeptides were renatured as above and pullulan-hydrolytic activity was observed as a clear band on a red background.

3.16 RNA extraction

RNA was extracted from various wheat tissues using the hot phenol method (Maes and Messens, 1992, Båga et al., 1995) with some modifications. The frozen wheat tissues (100 mg) were ground in liquid nitrogen. The ground tissues were incubated at 60°C for 5 min in 1:1 (v/v) mixture of acidic phenol and RNA extraction buffer (50 mM sodium acetate pH 4.5, 20 mM EDTA, 2% SDS, 50 mM β-mercaptoethanol). The slurry was centrifuged at 15,000 x g for 5 min to separate the upper aqueous phase from the organic phase. RNA in the aqueous phase was re-extracted at 60°C for 5 min with an equal amount of acidic phenol and centrifuged as described above. The RNA in the aqueous

phase was precipitated with 0.1 vol of 3 M sodium acetate pH 4.5 and 2.5 vol of cold ethanol, and pelleted by centrifugation at 15,000 x g for 10 minutes. The RNA pellet was washed twice with 3 M sodium acetate pH 5.2 followed with 80% ethanol. The pellet was dried and dissolved in diethyl pyrocarbonate (DEPC)-treated water. The RNA concentration was determined using spectrophotometer and calculated as follows:

RNA (μg/ml) = ($A_{260} - A_{320}$) x 40 x dilution factor

3.17 Reverse transcriptase (RT)-PCR

Total RNA extracted from leaf, root, shoot, and floret tissues of wheat was reverse transcribed using SUPERSCRIPT II RNase H^- reverse transcriptase (GIBCO BRL). The first strand cDNA synthesis reaction contained 2 μg total RNA, 5 μM random decamers (Ambion), 0.5 mM dNTPs, 10 mM DTT, and 200 units SUPERSCRIPT II RNase H- reverse transcriptase in 1x first strand buffer (50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$). The reaction (20 μl) was carried out at 42°C for 50 min, and 70°C for 15 min. One μl of the resulting solution was used as the template in a PCR reaction. Multiplex RT-PCR was performed to simultaneously amplify a 619-bp wheat isoamylase cDNA fragment and a 315-bp 18S rRNA fragment. Amplification of the 315-bp 18S rRNA fragment was used as an internal control for RT-PCR from different samples (Universal QuantumRNA 18S Internal Standards, Ambion). The multiplex RT-PCR reaction contained 0.2 mM dNTPs, 0.4 μM of each gene-specific primer, 66 nM 18S rRNA primer pair (Ambion), 932 nM 18S rRNA PCR competimers (Ambion), and 1.5 units *Taq* polymerase. The PCR was

performed as follows: 94°C 45 s, 60°C 1 min, 72°C 2 min for 25 cycles, and additional annealing at 72°C for 15 min.

3.18 3′-Rapid amplification of cDNA ends (3′-RACE)

Total RNA extracted from 5-dpa wheat kernels was reverse transcribed using oligo d(T)- anchor primer (5′-GACCACGCGTATCGATG TCGACTTTTTTTTTTTTTTT-3′; 5′/3′ RACE Kit, Boehringer Mannheim). The cDNA was amplified using a primer derived from the first conserved region of wheat isoamylase (5′-GGGATGTTGTCTTCAATCATAC-3′) and a PCR anchor primer (5′-GACCACGCGTATCGATGTCGAC-3′). The first strand RACE cDNA synthesis reaction contained 1 μg RNA extracted from 5 dpa wheat kernels, 1.9 μM oligo d(T)-anchor primer, 1 mM of each dNTP, and 20 units AMV reverse transcriptase (Boehringer Mannheim) in 1x RACE cDNA synthesis buffer (50 mM Tris-HCl, 8 mM $MgCl_2$, 30 mM KCl, 1 mM DTT pH 8.5). The reaction was incubated at 55°C for 60 min, and 65°C for 10 min. One μl of the resulting solution was used as the template in a PCR reaction. The PCR reaction contained 0.2 mM dNTPs, 0.4 μM gene-specific primer derived from the first conserved region of isoamylase, 0.4 μM PCR anchor primer, and 1.5 units *Taq* polymerase in 1x PCR buffer. The PCR was performed as follows: 94°C 45 s, 55°C 1 min, 72°C 2 min for 35 cycles, and additional annealing at 72°C for 15 min. The PCR products were cloned into the pCR2.1 vector as previously described. Transformed cells carrying different inserts were subjected to nucleotide sequencing.

3.19 Northern blot analysis

Total RNA (10 μg) extracted from developing and germinating kernels, and leaf, root, shoot, and floret tissues of wheat was fractionated on formaldehyde-containing agarose gel according to a method described by Sambrook et al. (1989). The gel contained 1.2% agarose and 2.2 M formaldehyde in 1x gel running buffer (20 mM MOPS pH 7.0, 8 mM sodium acetate, 1 mM EDTA pH 8.0). The RNA sample was denatured in the presence of 2.2 M formaldehyde and 11 M formamide at 65°C for 15 min. Two μl of loading buffer (50% glycerol, 1 mM EDTA pH 8.0, 0.25% bromophenol blue, 0.25% xylene cyanol FF) were added to each 20-μl RNA sample. The gel was run at 5 V/cm. After electrophoresis, the gel was submerged in DEPC-treated water for 2 x 10 min, and subsequently in 10x SSC (1.5 M NaCl, 150 mM sodium citrate pH 7.0) for 2 x 15 min. The RNA was blotted onto a positively charged nylon membrane (Hybond-N^+, Amersham) using vacuum transfer method at 70 psi for 2 h in 10 x SSC buffer. The RNA was crosslinked onto the membrane using 1.2 x 10^5 μJ of UV energy (UV Stratalinker, Stratagene). The membrane was air-dried and kept at 4°C until use.

3.20 Genomic DNA extraction

Wheat genomic DNA was extracted from 20-day old leaves using the modified CTAB method (Doyle and Doyle, 1990, Nair et al., 1997). Freeze-dried leaf material was ground into a fine powder in liquid nitrogen. The ground leaves were incubated at 60°C for 30 min in CTAB extraction buffer (4% CTAB, 1.4 M NaCl, 20 mM EDTA, 0.1 M Tris-HCl pH 8.0, 0.5% polyvinyl pyrrolidone

[PVP], 50 mM β-mercaptoethanol) followed by addition of an equal volume of chloroform:isoamyl alcohol (24:1 v/v) mixture. The aqueous phase was separated by centrifugation at 2,000 x g for 15 min, and re-extracted twice with an equal volume of 24:1 (v/v) chloroform/isoamyl alcohol mixture. The DNA in the aqueous phase was precipitated by adding 2/3 vol of ice-cold isopropanol, then pelleted by centrifugation at 3,000 x g for 30 min. The pellet was washed for 2 x 20 min in DNA wash buffer (70% ethanol, 10 mM ammonium acetate). The pellet was recovered by centrifugation at 3,000 x g for 15 min, air-dried for 3-5 min, and dissolved in TE buffer pH 8.0. The sample was treated with RNase (20-30 μg/ml) for 1 h at 37°C. The DNA was precipitated at -20°C for 30 min or overnight with 0.5 vol ammonium acetate (7.5 M) and 2.5 vol ethanol. The DNA was pelleted (15,000 x g for 15 min), washed twice with 70% ethanol, air-dried, and redissolved in TE buffer pH 8.0.

3.21 Southern blot analysis

Twenty microgram aliquots of wheat genomic DNA were digested with various restriction endonucleases, and then fractionated on 1% agarose gel. After eletrophoresis, the gel was submerged in 0.25 M HCl for 30 min, rinsed in distilled water, and then placed in denaturation buffer (1.5 M NaCl, 0.5 M NaOH) for 2 x 15 min. The gel was rinsed in distilled water and placed in neutralization buffer (1.5 M NaCl, 0.5 M Tris-HCl pH 7.2, 1 mM EDTA) for 2 x 15 min. The DNA was transferred onto a positively charged nylon membrane (Hybond-N^+, Amersham) using vacuum transfer method at 70 psi for 2 h in 10x

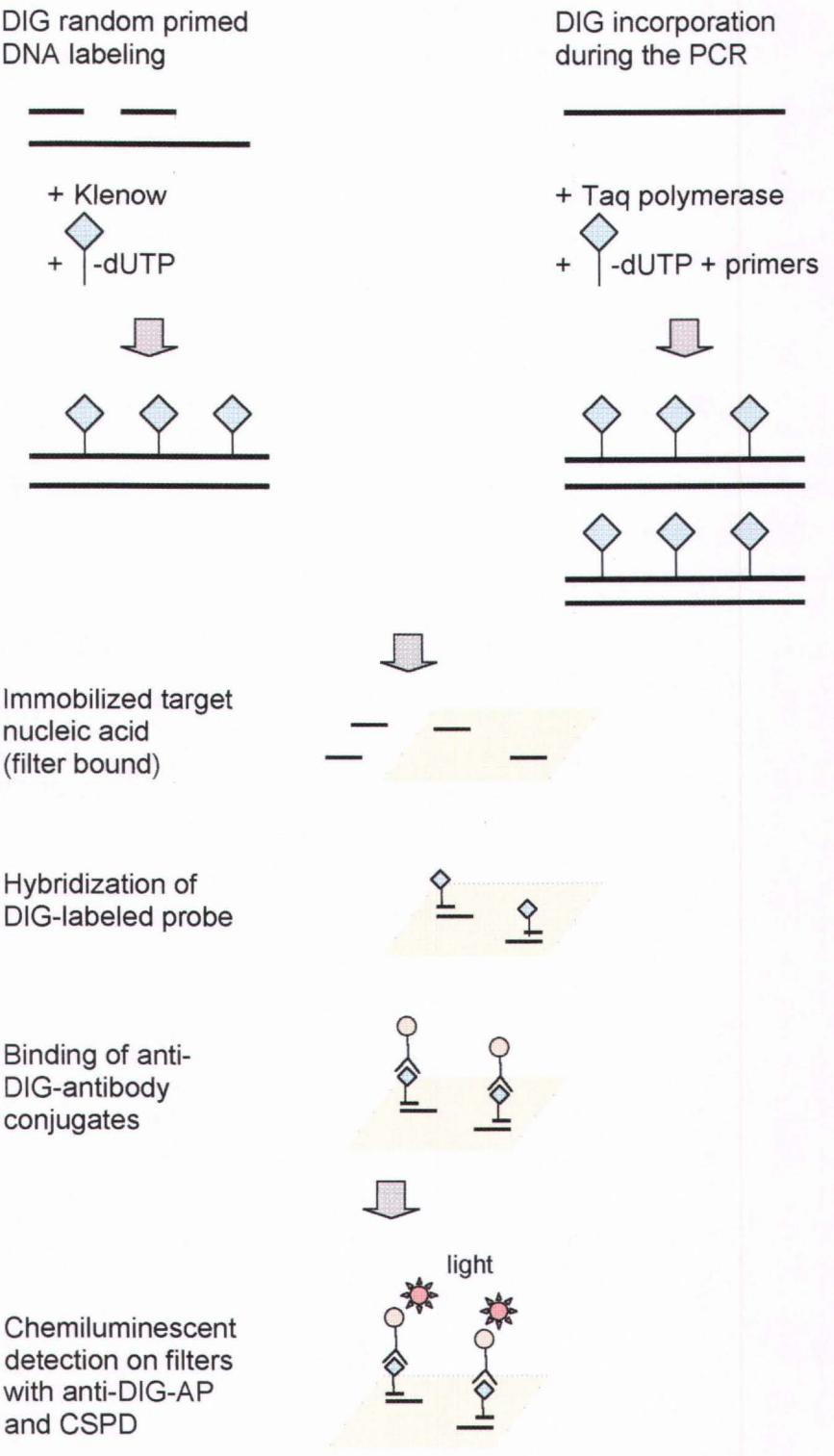
SSC buffer. The DNA was UV-crosslinked (1.2 x 10^5 μJ of UV energy; UV Stratalinker, Stratagene) and the membrane was air-dried.

3.22 Non-radioisotopic hybridization and signal detection

Hybridization, washes, and chemiluminescent signal detection were performed as described by Engler-Blum et al. (1993) (Figure 3.4). The membrane containing either DNA or RNA was incubated for at least 1 h at 68°C in prehybridization solution (0.25 M Na_2HPO_4 pH 7.2, 1 mM EDTA, 20% SDS, 0.5% blocking reagent [Boehringer Mannheim]). The DNA or RNA was hybridized overnight at 68°C with DIG-labeled DNA probe (2.5 ng probe/ml prehybridization solution). The 619-bp wheat isoamylase DNA fragment and the 2.6-kb full-length wheat cDNA, labeled with DIG-dUTP, were used as probes in Northern and Southern blot analyses, respectively. After hybridization, the membrane was washed at 65°C for 4 x 20 min with prewarmed wash buffer (20 mM Na_2HPO_4, 1 mM EDTA, 1%SDS). The following signal detection steps were performed at room temperature. The membrane was briefly washed for 5 min in DIG wash buffer (0.1 M maleic acid, 3 M NaCl, 0.3% Tween 20), and then incubated for 1 h in blocking buffer (DIG wash buffer containing 0.5% blocking reagent [Boehringer Mannheim]). The membrane was incubated for 30 min in conjugate buffer (blocking buffer containing 1:50,000 dilution of alkaline phosphatase-conjugated anti-DIG antibody [Boehringer Mannheim]). The membrane was washed for 4 x 10 min with DIG wash buffer. The positive signals were detected by incubating the membrane for 5 min with 0.24 mM CSPD (disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2′-(5′-chloro) tricyclo

[3.3.1.1^{3,7}]decan}-4-yl in 0.1 M Tris-HCl, 0.1 M NaCl, 50 mM $MgCl_2$ pH 9.5 buffer). The membrane was exposed to an X-ray film for a period of time depending on the signal intensity.

Figure 3.4 Northern and Southern blot analyses using DIG-labeled probes
The diagram outlines the steps in Northern and Southern blot analyses when a DIG-labeled DNA fragment is used as a probe. The horizontal lines represent nucleic acid strands. The symbols  represent DIG, alkaline phosphatase-conjugated anti-DIG antibody, and CSPD (a substrate of alkaline phosphatase), respectively.


DIG random primed
DNA labeling
+ Klenow
+
-dUTP
DIG incorporation
during the PCR
+ Taq polymerase
+
-dUTP + primers
Immobilized target
nucleic acid
(filter bound)
Hybridization of
DIG-labeled probe
Binding of anti-
DIG-antibody
conjugates
light
Chemiluminescent
detection on filters
with anti-DIG-AP
and CSPD

4. RESULTS AND DISCUSSION

4.1 Determination of starch concentration in wheat kernels

The hexaploid wheat (*Triticum aestivum* L.) cultivar CDC Teal was used for this study; CDC Teal belongs to the Hard Red Spring Wheat market class. As compared to most other wheat cultivars grown in Canada, CDC Teal is high yielding and high in protein (Hucl and Chibbar, 1996).

In this study, concentrations of starch in 5-, 10-, 15- and 20-dpa kernels and mature seeds were determined using an amylase/amyloglucosidase method (McCleary et al., 1994). Hydrolysis of starch with amylase and amyloglucosidase results in the formation of glucose. The amount of glucose released from each sample was determined using a coupled reaction catalyzed by glucose oxidase and peroxidase (McCleary and Codd, 1991). The concentration of starch was calculated by comparing the amounts of glucose released from the sample and from a glucose standard. To determine the concentration of amylose in wheat grains, the sample was first treated with Con A, and then processed in a similar manner as to determine the concentration of starch. The concentration of amylopectin in a sample was indirectly determined by subtracting the amount of amylose from the total starch concentration.

Only a small amount of starch (2.4 ± 0.8%) was observed in 5-dpa wheat kernels (Table 4-1). Almost half of the polysaccharide present at this stage was

linear molecules that could not complex with Con A. As the kernels matured, the amount of starch present in wheat grains gradually increased. The amount of amylose, on the other hand, decreased from 40 ± 8.2% in 5-dpa kernels to 17.6 ± 2.8% in 10-dpa kernels. The concentration of amylose was relatively stable as the kernels developed from 10 dpa to maturity. Because the concentration of amylose in a sample was expressed as a percentage of total starch, the results implied that the rate of amylose synthesis in 10-dpa kernels remained the same as that of starch synthesis until the kernels reached the fully matured stage. Unlike amylose, amylopectin was synthesized in small quantity when wheat kernels started to develop. Amylopectin was synthesized at a higher rate at the later stages of wheat kernel development.

Hucl and Chibbar (1996) reported that the concentration of starch, as determined by amylase/amyloglucosidase method, in mature kernels of the wheat cultivar CDC Teal was 67.4%. This is higher than the result observed in this study (51.5 ± 7.0%). Excluding the factors derived from different investigators and equipment, the difference of the results was most likely due to the moisture content of the samples. Whereas the samples used by Hucl and Chibbar (1996) were dried to a moisture content of 10 ± 1%, the samples used in this study were not subjected to a drying process. In addition, small variation of the results might result from environmental factors. While the samples in the study of Hucl and Chibbar (1996) were obtained from field, all plant material used in this study were grown in a greenhouse.

Table 4-1 Concentrations of starch and amylose in wheat kernels

Developmental stages of wheat kernels	% Starch (per 100 mg flour weight)	% Amylose (per total starch)
5 dpa	2.4 ± 0.8%	40.0 ± 8.2%
10 dpa	6.9 ± 3.6%	17.6 ± 2.8%
15 dpa	19.2 ± 0.4%	21.3 ± 3.3%
20 dpa	27.3 ± 2.5%	19.5 ± 1.7%
Mature	51.5 ± 7.0%	19.4 ± 2.9%

4.2 Amylolytic enzyme activities in developing wheat kernels

4.2.1 Limit dextrinase activity

Limit dextrinase activities at different stages of developing wheat kernels were determined using pullulan as the substrate. Two types of pullulan, colorless and dye-crosslinked forms, were used in this study. The insoluble, azurine-crosslinked pullulan was in a tablet form (Limit DextriZyme, Megazyme International Ireland Ltd.). According to the manufacturer, limit dextrinase activity is proportional to the soluble dyed product released from hydrolysis of the insoluble substrate. The second limit dextrinase assay used soluble pullulan as the substrate. The activity of limit dextrinase was calculated based on the amounts of reducing sugars released from pullulan.

Limit dextrinase from barley exists in two forms: a free or highly active form and a bound or less active form (Yamada, 1981). The two forms of barley limit dextrinase are convertible in the presence of reducing agents (McCleary, 1992, Cho et al., 1999). Increase in activity and stability of barley limit dextrinase was reported in extracts containing DTT (MacGregor et al., 1994, Sissons, 1996). Prior to determination of enzymatic activity, limit dextrinase must be activated during protein extraction in the presence of buffers containing DTT and no protease inhibitors (Lee and Pyler, 1984). In this study, extraction of limit dextrinase at 40°C, as described by McCleary (1992), did not yield higher limit dextrinase activity as compared to extraction at 4°C. To minimize protein degradation and to maintain enzymatic activities, extraction of limit dextrinase in this study was performed at 4°C.

To optimize the conditions used in limit dextrinase assays, limit dextrinase activities were determined at various protein concentrations and incubation time. Subsequent enzyme assays were performed at conditions where limit dextrinase activity increased linearly with the protein concentration and incubation time. When using dye-crosslinked pullulan as the substrate, limit dextrinase activity was linear in the range of 0.2-1.0 μg/μl total protein concentration (Figure 4.1) and 0-60 min incubation time (Figure 4.2). Limit dextrinase extracted from developing wheat kernels showed highest activity at pH 5.0 (Figure 4.3), which is similar to limit dextrinases from developing maize kernels (Doehlert and Knutson, 1991) and spinach leaf (Henker et al., 1998). To determine limit dextrinase activities at different stages of developing wheat kernels, the samples were adjusted to 0.5 μg/μl total protein concentration. The enzyme assays were performed by incubating the samples for exactly 20 min at pH 5.0.

During wheat kernel development, highest activity of limit dextrinase was observed at 15 dpa (Figure 4.4). The activity decreased at later stages; however, low activity of limit dextrinase, as measured by Limit DextriZyme assay, could still be observed in mature wheat kernels (Figure 4.4B). This activity might represent the bound form of limit dextrinase, similar to that observed in barley (McCleary, 1992).

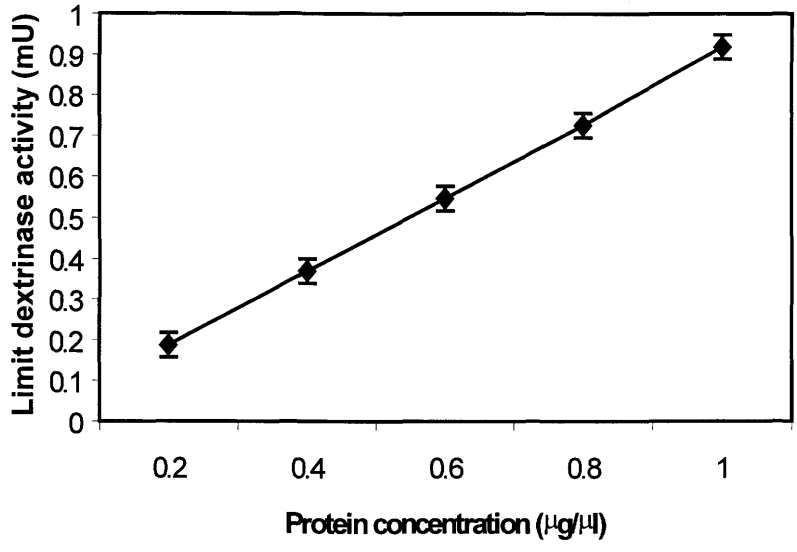


Figure 4.1 Relationship between limit dextrinase activity and total protein concentration

Limit dextrinase was extracted from developing wheat endosperm. The extracts were adjusted to contain 0.2 to 1.0 μg/μl total protein concentration. The activity of limit dextrinase was determined using dye-crosslinked pullulan as the substrate. The standard error bars indicate variation of the results obtained from independent experiments, each with at least three replications.

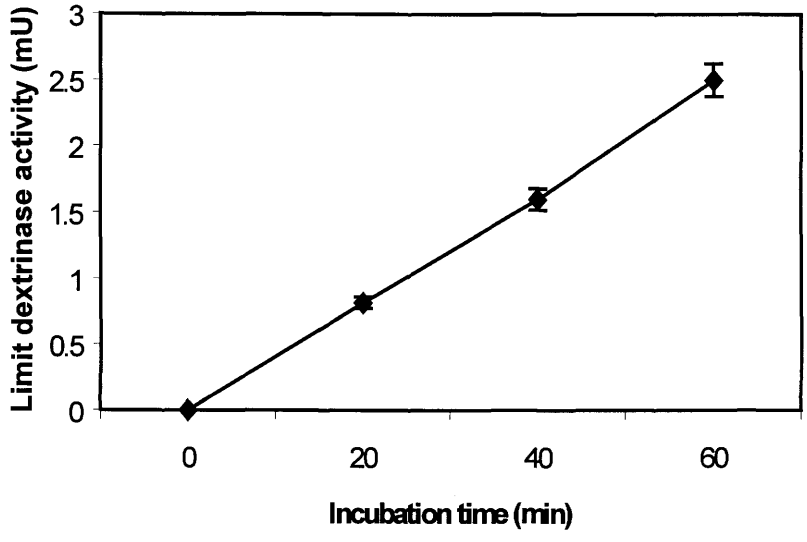
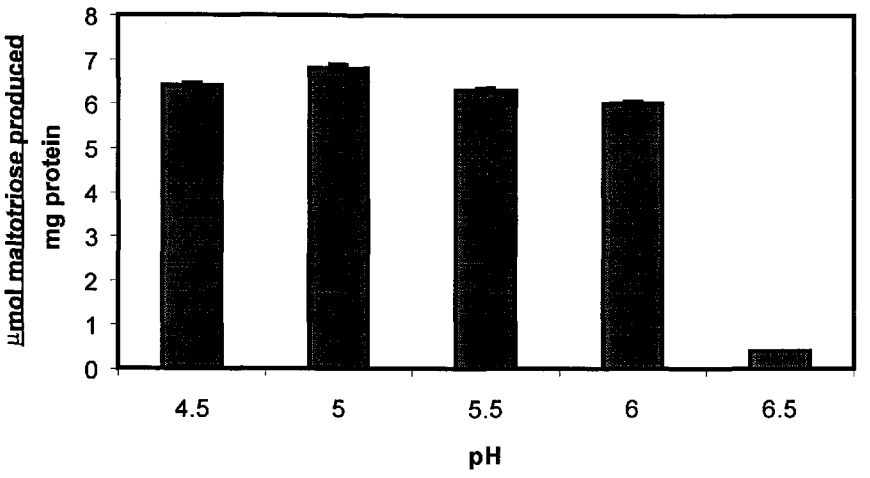


Figure 4.2 Effect of incubation time on limit dextrinase activity
Limit dextrinase activity was determined using dye-crosslinked pullulan as the substrate. The incubation time was varied from 0 to 60 min, while the protein concentration was maintained at 0.5 μg/μl. Within a range of incubation time tested, linear activity of limit dextrinase was observed. The standard error bars indicate variation of the results obtained from independent experiments, each with at least three replications.

A.



B.

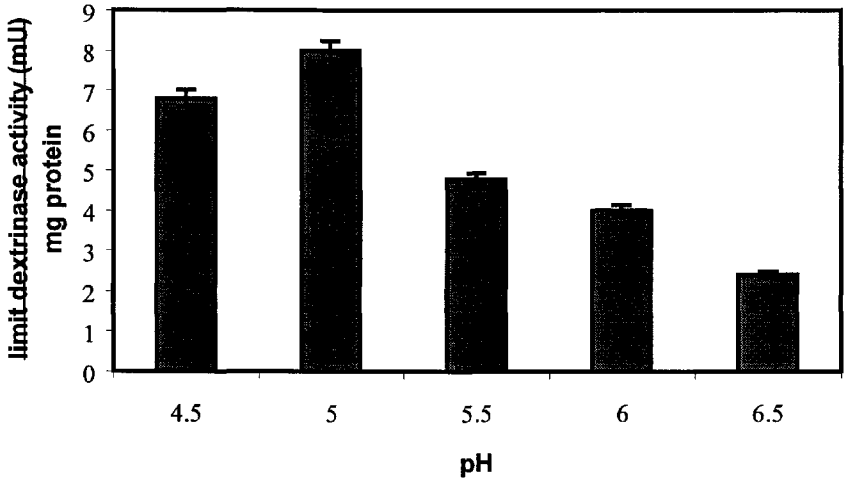
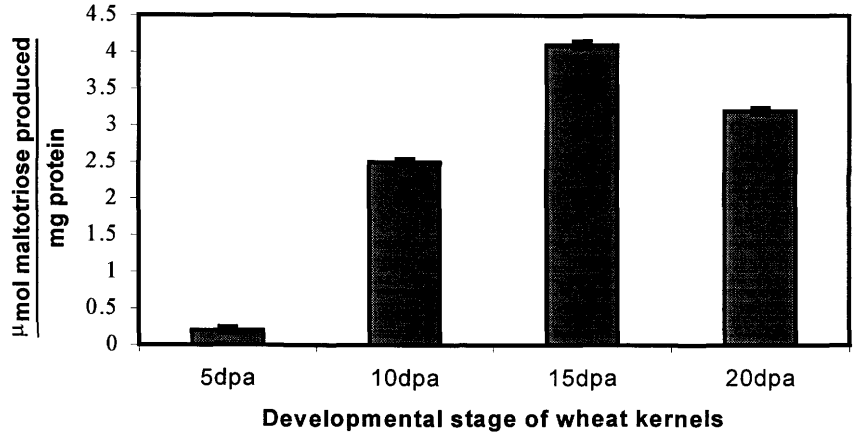


Figure 4.3 Optimal pH for wheat limit dextrinase

Limit dextrinase activity was determined at various pHs in the presence of pullulan (A) or dye-crosslinked pullulan (B) as the substrate. Both assays were performed at 40°C for 20 min in the presence of 0.5 µg/µl total protein concentration. In both assays, the highest activity of limit dextrinase was observed at pH 5.0. The standard error bars indicate variation of the results obtained from independent experiments, each with at least three replications.

A.



B.

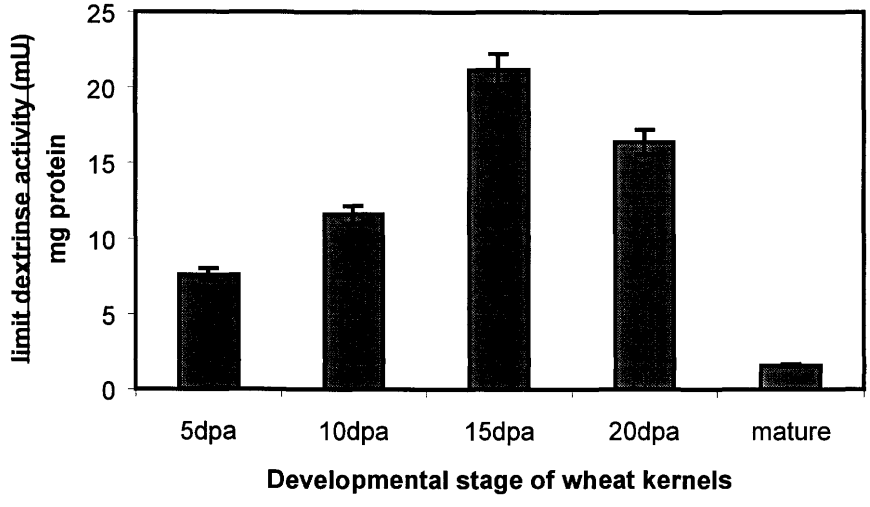


Figure 4.4 Limit dextrinase activity in developing wheat grains
Limit dextrinase was extracted from 5-, 10-, 15-, 20-dpa, and mature wheat kernels. The enzyme assays were performed using pullulan (A) or dye-crosslinked pullulan (B) as the substrate. The standard error bars indicate variation of the results obtained from independent experiments, each with at least three replications.

The results in this study showed that limit dextrinase activity is associated with the amount of starch present in wheat grains (Table 4-1, Figure 4.4). In 5-dpa kernels, where a small amount of starch was accumulated, low activity of limit dextrinase was observed (Figure 4.4). The activities of limit dextrinase and concentrations of starch increased as the kernels matured. As compared to the high concentration of amylose in 5-dpa kernels, amylopectin was observed at the later stages of wheat kernel development. According to Pan and Nelson (1984), amylopectin synthesis requires a balance between branching and debranching enzyme activities. According to expression patterns of SBEs in 15-dpa wheat kernels, total starch branching enzyme activities are predicted to derive mainly from SBEI and SBEIc (Repellin et al., 1997, Båga et al., 2000), with only a small quantity from SBEII (Nair et al., 1997). Therefore, the results suggest that limit dextrinase activity is required to counterbalance the activities of SBEI and SBEIc and this is essential to maintain a constant ratio of amylose and amylopectin in developing wheat kernels.

4.2.2 Total amylolytic activity

Limit dextrinase activities at different stages of developing wheat kernels could be determined using pullulan as the substrate. On the other hand, α-1,4- and α-1,6-degrading enzymes present in the extracts could interfere with isoamylase activity when using amylopectin as the substrate. Therefore, the activity obtained was referred to as total amylolytic activity. However, subtraction of limit dextrinase activity from total amylolytic activity would give an estimate of isoamylase activity in the extracts.

Hydrolysis of amylopectin with various amylolytic enzymes would produce a mixture of dextrin, oligosaccharides, and monosaccharides as the products. If the total amylolytic activity was determined by measuring the amounts of reducing sugars released, total amylolytic activity would be contributed mainly from amylases. Hydrolysis of amylopectin with α-1,4-degrading enzymes results in the formation of oligo- and monosaccharides, which results in the production of high reducing values. In this study, the total amylolytic activity was determined by measuring the increase in the absorbance of the iodine-amylopectin complex. The oligo- and monosaccharides obtained from high amylase activities do not bind iodine and, thus, do not result in the overestimation of total amylolytic activity present in plant extracts. Therefore, the total amylolytic activity, as determined by the increase in the absorbance of the iodine-amylopectin complex at 550 nm, was contributed from both α-1,4- and α-1,6-degrading enzymes.

Total amylolytic activities were determined at different stages of developing wheat kernels. Highest activity of amylolytic enzymes was observed in 15-dpa kernels (Figure 4.5). This result correlates with the activity of limit dextrinase present in wheat grains (Figure 4.4). However, the activities of amylolytic enzymes observed during wheat kernel development may be due to limit dextrinase, isoamylase, and/or other α-1,4-degrading enzymes. To better understand the role of isoamylase in wheat starch synthesis, a cDNA encoding isoamylase in wheat was isolated and characterized.

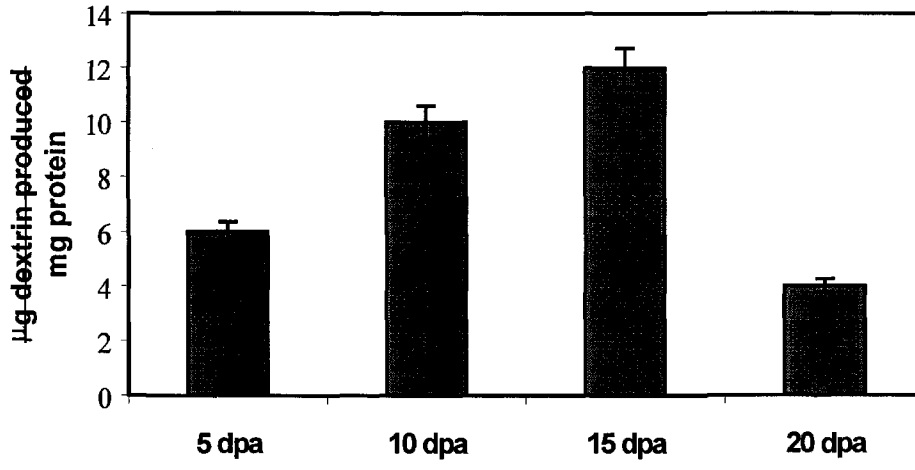


Figure 4.5 Total amylolytic activity in developing wheat grains
Total amylolytic activities in 5-, 10-, 15, and 20-dpa wheat kernels were determined using amylopectin as the substrate. The standard error bars indicate variation of the results obtained from independent experiments, each with at least three replications.

4.3 Isolation of a full-length isoamylase cDNA from wheat

4.3.1 Synthesis of an isoamylase DNA probe

Because isoamylase activity can not be accurately determined from total plant extracts, this project therefore focused on molecular characterization of an isoamylase cDNA in developing wheat kernels. A partial isoamylase DNA fragment was amplified from a cDNA library constructed from 12-dpa wheat kernel poly (A)+ RNA (Nair et al., 1997). The amplified product was used as a probe to isolate a full-length isoamylase cDNA from a wheat cDNA library.

According to the deduced amino acid sequence of maize isoamylase mRNA (James et al., 1995, GenBank accession number U18908), the four conserved regions essential for catalytic activity of isoamylase span the middle region of the 2,712-bp transcript, occupying nucleotide 1111 to 1725. Two oligonucleotide primers used to amplify a partial isoamylase DNA fragment from a wheat cDNA library were designed based on the first and fourth conserved regions of maize isoamylase. The primers cover the region from nucleotide 1110 to nucleotide 1728 of the maize transcript. Therefore, if the primers could anneal with wheat cDNA templates and the lengths within that region of wheat and maize isoamylase cDNA are similar, the PCR would yield a 619-bp product. Ten different pools of total 48 wheat pools, obtained from independent transformations (Nair et al., 1997), cDNA library were randomly selected and used as templates in PCR. After 30 cycles of PCR at annealing temperature of 55°C, an amplified product of approximately 600 bp was observed from every library pool tested (Figure 4.6).

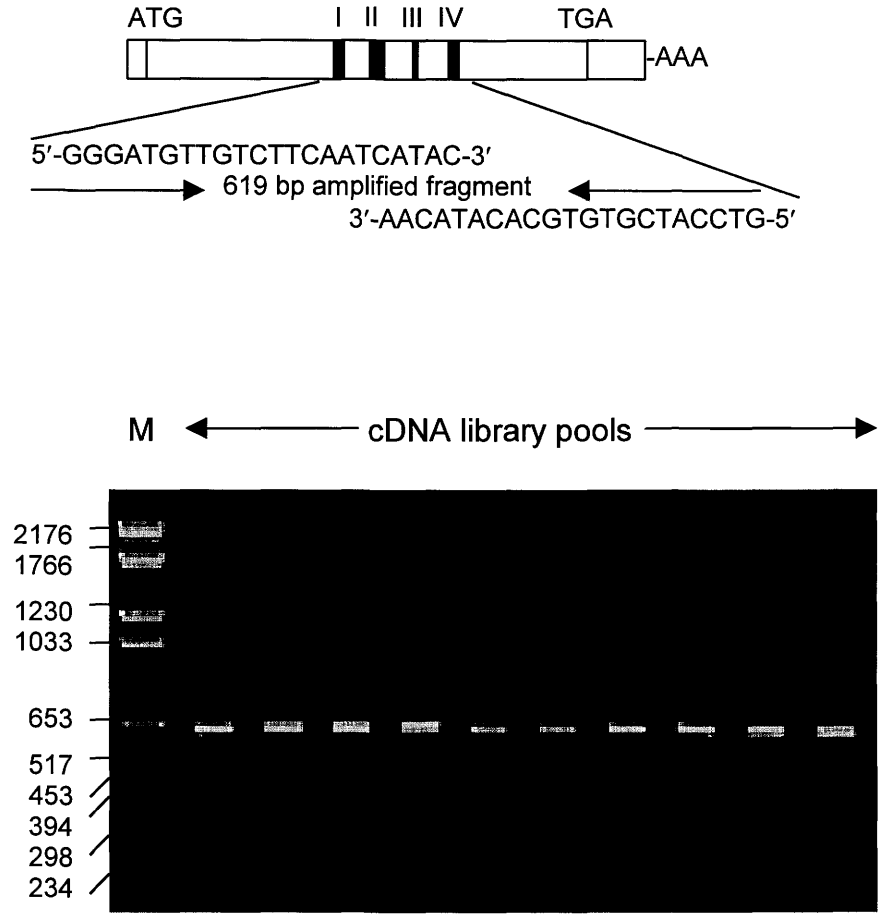


Figure 4.6 Amplification of a partial isoamylase DNA fragment
The diagram shows schematic picture of maize cDNA (James et al., 1995), which carries four conserved regions of isoamylase (as shown in Roman numbers). Two oligonucleotide primers were synthesized based on the first and fourth conserved regions of maize. Ten cDNA library pools constructed from 12-dpa wheat kernel poly(A)+ RNA (Nair et al., 1997) were used as templates in PCR. A 619-bp amplified product was observed in all library pools tested. The sizes in base pairs of molecular weight marker (M) are indicated on the left.

According to the action of *Taq* polymerase, a single non-template directed deoxyadenosine residue would be added to the 3′ end of PCR products. Therefore, the amplified fragments could be cloned into pCR2.1 vectors (Invitrogen), which carry 5′ d(T)-overhang at the cloning site (Marchuk et al., 1991). Insertion of foreign DNA into the pCR2.1 vector disrupts continuous coding sequence of the functional fragment of β-galactosidase (*lacZα*) gene. Transformed cells carrying recombinant pCR2.1 vectors were therefore selected based on blue-white colony appearance in the presence of X-gal. The recombinant pCR2.1 vectors were transformed into *E.coli* INVαF′ cells. Transformed cells were grown on LB-kanamycin plates with X-gal spread on the media surface and the cells carrying recombinant plasmids were selected based on white colony appearance. Plasmids isolated from a number of white colonies were further analyzed.

According to the restriction map of pCR2.1 vectors (Figure 3.1), the cloning site of pCR2.1 is flanked with two EcoRI sites. Digestion of the recombinant pCR2.1 vectors with EcoRI yielded two DNA fragments; a 3.9-kb fragment of pCR2.1 vector and a 600-bp fragment of wheat DNA insert (Figure 4.7). To determine the identity of the wheat DNA insert, ten recombinant pCR2.1 vectors carrying wheat DNA fragments were purified and then subjected to nucleotide sequence analysis. The results confirmed that all the wheat DNA fragments were 619 bp long. Amino acid sequence comparisons using the program BLAST (Altschul et al., 1990) revealed that the 619-bp fragment has high degree of similarity to isoamylases from various organisms.

The 619-bp wheat DNA fragment showed 88% similarity with nucleotide sequence in the middle region of maize isoamylase cDNA where the four conserved regions of isoamylase are encoded (nucleotide 1110 to 1728) (Figure 4.8). The 619-bp fragment also showed low degree of amino acid sequence similarity to limit dextrinases, pullulanases, glycogen debranching enzymes, and starch branching enzymes, all of which are members of the α-amylase family (Svensson, 1994). From DNAstar software, deduced amino acid sequence of the 619-bp wheat DNA fragment showed 92-98%, 25-33%, 16-18%, 17-18%, 12-13% similarity to plant isoamylases, bacterial isoamylases, limit dextrinases, pullulanases, and glycogen debranching enzymes from various sources, respectively. The results thus indicate that the 619-bp DNA fragment amplified from the developing wheat kernel cDNA library encodes isoamylase.

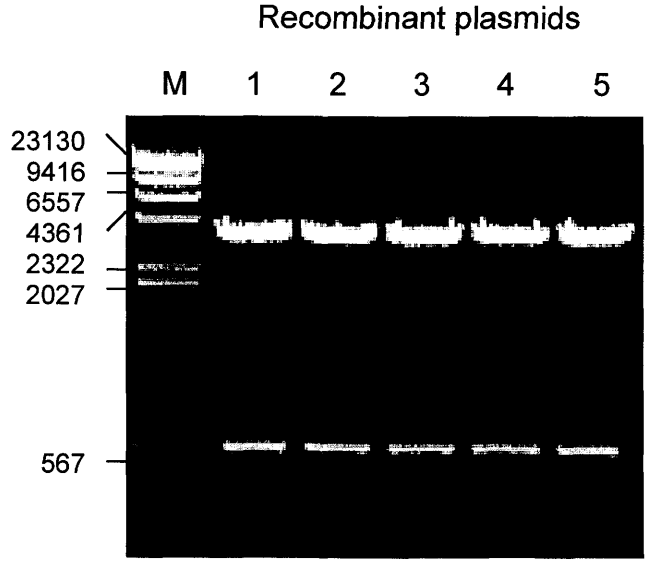


Figure 4.7 EcoRI digestion of recombinant pCR2.1 vectors
Wheat DNA fragments of approximately 600 bp were cloned into pCR 2.1 vectors. Digestion of five recombinant plasmids with EcoRI yielded a 3.9-kb fragment of pCR 2.1 vector and a 619-bp fragment of wheat DNA insert. The sizes in base pairs of molecular weight marker (M) are shown on the left.

```
MAIZE   GGATGTTGTCTTCAATCATACAGCTGAAGGTAATGAGAAAGGCCCAATAT
WHEAT   **************************G***G*******T**T*******

MAIZE   TATCCTTTAGGGGGATAGATAATAGTACATACTACATGCTTGCACCTAAG
WHEAT   ****A*********G*C*******C*********T***********C***

MAIZE   GGAGAGTTTTATAATTATTCTGGTTGTGGAAATACCTTCAATTGTAATCA
WHEAT   **************C********C*****G**********C********

MAIZE   TCCTGTAGTCCGTGAATTTATAGTGGATTGCTTGAGATACTGGGTAACAG
WHEAT   ******G**T***C****C**T**A*****T**A***********G**G*

MAIZE   AAATGCATGTTGATGGTTTTCGTTTTGACCTTGCATCTATACTGACCAGA
WHEAT   ****************************T********C***A********

MAIZE   GGATGCAGTCTATGGGATCCAGTTAATGTGTATGGAAGTCCAATGGAAGG
WHEAT   **T*C******G*************C*********GC******A*****

MAIZE   TGACATGATTACGACAGGGACACCTCTTGTTGCCCCACCACTTATTGACA
WHEAT   *********C**A******************A*T****************

MAIZE   TGATTAGCAATGACCCAATTCTTGGAAATGTCAAGCTCATTGCTGAAGCA
WHEAT   ****C**********************GGC*********************

MAIZE   TGGGATGCAGGAGGTCTCTATCAAGAAGGTCAGTTTCCTCACTGGAACGT
WHEAT   ********G*****C**********T******A**C***********T**

MAIZE   TTGGTCAGAGTGGAATGGAAAGTATCGCGATACCGTGCGTCAGTTCATCA
WHEAT   ******T***********G*****C**G**C*TT*****C**A*****T*

MAIZE   AAGGCACAGATGGATTTGCTGGTGCTTTTGCTGAATGCCTATGTGGAAGT
WHEAT   *******T****************G******C*****T**T*********

MAIZE   CCACAGTTATACCAGGCAGGGGGGAGGAAGCCTTGGCACAGTATCGGCTT
WHEAT   *****CC*************A**A*****A***************AA***

MAIZE   TGTATGTGCACACGATGGA
WHEAT   ******************
```

Figure 4.8 Alignment of isoamylase DNA fragments

A region of maize isoamylase cDNA (nucleotide 1110 to 1728) (James et al., 1995, GenBank accession no. U18908) was aligned with a 619-bp wheat DNA fragment using DNAstar software. Identical nucleotides are shown in asterisks and variable nucleotides at the corresponding location are indicated. The regions encoding four conserved regions of isoamylase were indicated in bold.

4.3.2 Characteristics of a full-length isoamylase cDNA

A pCR2.1 vector carrying a 619-bp wheat DNA fragment was digested with EcoRI. The EcoRI-treated wheat DNA fragment (635 bp long) was randomly labeled with [^{32}P]-dCTP to generate a radiolabeled probe, which was consequently used to screen the cDNA library constructed from 12-dpa wheat kernel poly (A)+ RNA (Nair et al., 1997). Screening of 1 x 10^6 pfu wheat cDNA library with a partial wheat isoamylase DNA fragment yielded 20 positive clones, ranging in size from 0.7 to 2.6 kb. Restriction patterns showed that most of the clones varied in length at the 5′-regions. Therefore, the clone carrying a 2.6-kb wheat cDNA was further characterized (Figure 4.9). It was also observed that, as compared to the other 19 positive clones, only one cDNA clone carried a 1.5-kb wheat cDNA that lacks most of the region at the 3′ end. The characteristics of the 1.5-kb wheat cDNA will be discussed.Most plant mRNAs carry a short 5′-untranslated region, usually <100 nucleotides (Bailey-Serres, 1999). In the coding region of the 2,590-bp wheat cDNA, the first 5′-ATG and the stop codon (TGA) were observed at nucleotide 14 and 2384, respectively (Figure 4.9). A sequence with similarity to the consensus polyadenylation signal AATAAA was present 125 bp upstream of the poly (A)+ signal. Additionally, a cluster of three more in-frame start codons was observed within 15 bp downstream of the first ATG. Kozak (1997) suggested that the most conserved residue at the translation initiation site is purine, usually A at the –3 position and G at +4 position (when A of the ATG start codon is numbered +1 position). According to the putative context sequences at the translation initiation site of

plant genes (Joshi et al., 1997), the context sequences of four start codons of wheat isoamylase cDNA do not follow the putative sequence at the translation initiation site of genes in monocot plant. However, the third ATG located at nucleotide 23 most likely represents the efficient start codon of isoamylase transcript in wheat. The polypeptide deduced from the third start codon also shows an important characteristic of chloroplast proteins in which the residue adjacent to the initiator methionine is almost invariably an alanine (von Heijne et al., 1989).

Translation in eukaryotic cells is usually initiated at the first start codon located at the 5′-end of the mRNA (Cigan et al., 1988). However, it has also been indicated that translation initiation complex possibly screens all the start codons in the 5′-region (Kozak, 1991). For efficient translation, the translation initiation complex would pass the start codon with poor context sequence even though it is the first that the complex encounters. On the other hand, translation does not always occur at the most efficient start codon. Fütterer and Hohn (1996) indicated that translation at a start codon with poor context sequence played a role to regulate availability of proteins *in vivo*. According to the nucleotide sequence of wheat isoamylase cDNA, the GC-rich region downstream of the start codons possibly functions in stopping translation initiation complex at the first start codon, thus enhancing translation efficiency (Pain, 1996). Since it remains to be proven which start codon is responsible for translation initiation of isoamylase in wheat, the amino acid sequence of wheat isoamylase in this study is deduced from the first start codon located at

nucleotide 14. Therefore, the coding region of the 2,590-bp wheat isoamylase cDNA contains 2,370 nucleotides, coding for a polypeptide with a predicted molecular mass of 88 kDa (Figure 4.9).

Starch is synthesized in plastids, either in chloroplasts of leaves or amyloplasts of storage tissues. To function in starch biosynthesis, starch synthetic enzymes that are synthesized in cytosol must be imported into plastids. All starch synthetic enzymes known to date are synthesized as pre-proteins carrying a plastidal transit peptide at the N-terminus. The transit peptide is cleaved during or after translocation to produce a mature polypeptide chain in the target location (Keegstra, 1989). Intracellular localization studies of isoamylase in developing pea embryo (Zhu et al., 1998) and immature maize kernels (Yu et al., 1998) have confirmed that isoamylase is largely confined to the amyloplasts. Based on the deduced amino acid sequence of wheat isoamylase (Figure 4.9), a putative cleavage site motif of chloroplast transit peptide was observed between Ala^{51} and Ala^{52} (Gavel and von Heijne, 1990). Studies of nuclear-encoded chloroplast proteins has found that the transit peptides usually contain high content of serine and/or threonine residues and very few acidic amino acids (von Heijne et al., 1989). As compared to the transit peptides of other starch synthetic enzymes from wheat (Ainsworth et al., 1993, Li et al., 1999b, Rahman et al., 1999, Båga et al., 2000), the number of serine and threonine residues within the predicted transit peptide of wheat isoamylase is not very high (3 residues from total 51 amino acids). However, as compared to the number of those residues found within the mature isoamylase the

percentage of hydroxylated amino acid residues is higher within the expected transit peptide region than in the mature protein (17.6% as compared to 9.0% in mature protein). The frequency of acidic amino acids observed within the predicted transit peptide region and in isoamylase mature protein were 3.9% and 12.3%, respectively. In addition, the predicted region of wheat isoamylase transit peptide follows major characteristics of chloroplast transit peptides from various botanical sources. These include uncharged N-terminal domain, positively-charged central domain, and C-terminal domain with a high potential for forming amphiphilic β-strand (von Heijne et al., 1989). According to Karlin-Neumann and Tobin (1986), the GXGRV motif (where X is a variable amino acid residue) is usually observed in the C-terminal domain of chloroplastic transit peptide before the cleavage site. A somewhat similar, although not identical, motif GVGEV was found within the C-terminal domain of the predicted transit peptide of wheat isoamylase (Figure 4.9). The results indicate that the (V/I)-X-(A/C)-A motif (where cleavage occurs at the N-terminus of the last alanine residue) would most likely represent the transit peptide cleavage site of isoamylase in wheat (Gavel and von Heijne, 1990). Therefore, cleavage of the 51-amino acid transit peptide would yield 83-kDa mature isoamylase in amyloplasts of wheat kernels. The size of polypeptide predicted from the cDNA correlates with the size of protein extracted from developing wheat kernels (Figure 4.15).

```
CGGGGTTCCCGGCATGACAATGATGGCCATGCCCGAGGCGCCCTGCCTCT   50
              M  T  M  M  A  M  P  E  A  P  C  L

GCGCGCGCCCGTCCCTCGCCGCGCGCGCGAGGCGGCCGGGGTCGGGGCCG  100
C  A  R  P  S  L  A  A  R  A  R  R  P  G  S  G  P

GCGCCGCGCCTGCGACGGTGGCGACCCAATGCGACGGCGGGGAAGGGGGT  150
 A  P  R  L  R  R  W  R  P  N  A  T  A  G  K  G  V

CGGCGAGGTGTGCGCCGCGGTTGTCGAGGCGGCGACGAAGGTAGAGGACG  200
  G  E  V  C  A▼A  V  V  E  A  A  T  K  V  E  D

AGGGGGAGGAGGACGAGCCGGTGGCGGAGGACAGGTACGCGCTCGGCGGC  250
E  G  E  E  D  E  P  V  A  E  D  R  Y  A  L  G  G

GCGTGCAGGGTGCTCGCCGGAATGCCCGCGCCGCTGGGCGCCACCGCGCT  300
 A  C  R  V  L  A  G  M  P  A  P  L  G  A  T  A  L

CGCCGGCGGGGTCAATTTCGCCGTCTATTCCGGCGGAGCCACCGCCGCGG  350
  A  G  G  V  N  F  A  V  Y  S  G  G  A  T  A  A

CGCTCTGCCTCTTCACGCCAGAAGATCTCAAGGCGGATAGGGTGACCGAG  400
A  L  C  L  F  T  P  E  D  L  K  A  D  R  V  T  E

GAGGTTCCCCTTGACCCCCTGATGAATCGGACCGGGAACGTGTGGCATGT  450
 E  V  P  L  D  P  L  M  N  R  T  G  N  V  W  H  V

CTTCATCGAAGGCGAGCTGCACAACATGCTTTACGGGTACAGGTTCGACG  500
  F  I  E  G  E  L  H  N  M  L  Y  G  Y  R  F  D

GCACCTTTGCTCCTCACTGCGGGCACTACCTTGATGTTTCCAATGTCGTG  550
G  T  F  A  P  H  C  G  H  Y  L  D  V  S  N  V  V

GTGGATCCTTATGCTAAGGCAGTGATAAGCCGAGGGGAGTATGGTGTTCC  600
 V  D  P  Y  A  K  A  V  I  S  R  G  E  Y  G  V  P

AGCGCGTGGTAACAATTGCTGGCCTCAGATGGCTGGCATGATCCCTCTTC  650
  A  R  G  N  N  C  W  P  Q  M  A  G  M  I  P  L

CATATAGCACGTTTGATTGGGAAGGCGACCTACCTCTAAGATATCCTCAA  700
P  Y  S  T  F  D  W  E  G  D  L  P  L  R  Y  P  Q

AAGGACCTGGTAATATATGAGATGCACTTGCGTGGATTCACGAAGCATGA  750
 K  D  L  V  I  Y  E  M  H  L  R  G  F  T  K  H  D

TTCAAGCAATGTAGAACATCCGGGTACTTTCATTGGAGCTGTGTCGAAGC  800
  S  S  N  V  E  H  P  G  T  F  I  G  A  V  S  K

TTGACTATTTGAAGGAGCTTGGAGTTAATTGTATTGAATTAATGCCCTGC  850
L  D  Y  L  K  E  L  G  V  N  C  I  E  L  M  P  C
```

```
CATGAGTTCAACGAGCTGGAGTACTCAACCTCTTCTTCCAAGATGAACTT  900
 H  E  F  N  E  L  E  Y  S  T  S  S  S  K  M  N  K

TTGGGGATATTCTACCATAAACTTCTTTTCACCAATGACAAGATACACAT  950
 W  G  Y  S  T  I  N  F  F  S  P  M  T  R  Y  T

CAGGCGGGATAAAAAACTGTGGGCGTGATGCCATAAATGAGTTCAAAACT 1000
S  G  G  I  K  N  C  G  R  D  A  I  N  E  F  K  T

TTTGTAAGAGAGGCTCACAAACGGGGAATTGAGGTGATCCTGGATGTTGT 1050
 F  V  R  E  A  H  K  R  G  I  E  V  I  L  D  V  V

CTTCAACCATACAGCTGAGGGTAATGAGAATGGTCCAATATTATCATTTA 1100
   F  N  H  T  A  E  G  N  E  N  G  P  I  L  S  F

GGGGGGTCGATAATACTACATACTATATGCTTGCACCCAAGGGAGAGTTT 1150
R  G  V  D  N  T  T  Y  Y  M  L  A  P  K  G  E  F

TATAACTATTCTGGCTGTGGGAATACCTTCAACTGTAATCATCCTGTGGT 1200
 Y  N  Y  S  G  C  G  N  T  F  N  C  N  H  P  V  V

TCGTCAATTCATTGTAGATTGTTTAAGATACTGGGTGACGGAAATGCATG 1250
   R  Q  F  I  V  D  C  L  R  Y  W  V  T  E  M  H

TTGATGGTTTTCGTTTTGATCTTGCATCCATAATGACCAGAGGTTCCAGT 1300
V  D  G  F  R  F  D  L  A  S  I  M  T  R  G  S  S

                                   →
CTGTGGGATCCAGTTAACGTGTATGGAGCTCCAATAGAAGGTGACATGAT 1350
 L  W  D  P  V  N  V  Y  G  A  P  I  E  G  D  M  I

CACAACAGGGACACCTCTTGTTACTCCACCACTTATTGACATGATCAGCA 1400
   T  T  G  T  P  L  V  T  P  P  L  I  D  M  I  S

ATGACCCAATTCTTGGAGGCGTCAAGCTCATTGCTGAAGCATGGGATGCA 1450
N  D  P  I  L  G  G  V  K  L  I  A  E  A  W  D  A

GGAGGCCTCTATCAAGTAGGTCAATTCCCTCACTGGAATGTTTGGTCTGA 1500
 G  G  L  Y  Q  V  G  Q  F  P  H  W  N  V  W  S  E

GTGGAATGGGAAGTACCGGGACATTGTGCGTCAATTCATTAAAGGCACTG 1550
   W  N  G  K  Y  R  D  I  V  R  Q  F  I  K  G  T

ATGGATTTGCTGGTGGTTTTGCCGAATGTCTTTGTGGAAGTCCACACCTA 1600
D  G  F  A  G  G  F  A  E  C  L  C  G  S  P  H  L

TACCAGGCAGGAGGAAGGAAACCTTGGCACAGTATCAACTTTGTATGTGC 1650
 Y  Q  A  G  G  R  K  P  W  H  S  I  N  F  V  C  A

ACATGATGGATTTACACTGGCTGATTTGGTAACATATAATAAGAAGTACA 1700
   H  D  G  F  T  L  A  D  L  V  T  Y  N  K  K  Y
```

ATTTACCAAATGGGGAGAACAACAGAGATGGAGAAAATCACAATCTTAGC 1750
N L P N G E N N R D G E N H **N L S**

TGGAATTGTGGGGAGGAAGGAGAATTCGCAAGATTGTCTGTCAAAAGATT 1800
W N C G E E G E F A R L S V K R L

GAGGAAGAGGCAGATGCGCAATTTCTTTGTTTGTCTCATGGTTTCTCAAG 1850
R K R Q M R N F F V C L M V S Q

GAGTTCCAATGTTCTACATGGGTGATGAATATGGCCACACAAAAGGGGGC 1900
G V P M F Y M G D E Y G H T K G G

AACAACAATATATACTGCCATGATTCTTATGTCAATTATTTTCGCTGGGA 1950
N N N I Y C H D S Y V N Y F R W D

TAAAAAAGAACAATACTCTGAGTTGCACCGATTCTGCTGCCTCATGACCA 2000
K K E Q Y S E L H R F C C L M T

AATTCCGCAAGGAGTGCGAGGGTCTTGGCCTTGAGGACTTTCCAACGGCC 2050
K F R K E C E G L G L E D F P T A

AAACGGCTGCAGTGGCATGGTCATCAGCCTGGGAAGCCTGATTGGTCTGA 2100
K R L Q W H G H Q P G K P D W S E

GAATAGCCGATTCGTTGCCTTTTCCATGAAAGATGAAAGACAGGGCGAGA 2150
N S R F V A F S M K D E R Q G E

TCTATGTGGCCTTCAACACCAGCCACTTACCGGCCGTTGTTGAGCTCCCA 2200
I Y V A F N T S H L P A V V E L P

GAGCGCGCAGGGCGCCGGTGGGAACCGGTGGTGGACACAGGCAAGCCAGC 2250
E R A G R R W E P V V D T G K P A

ACCATACGACTTCCTCACCGACGACTTACCTGATCGCGCTCTCACCATAC 2300
P Y D F L T D D L P D R A L T I

ACCAGTTCTCGCATTTCCTCTACTCCAACCTCTACCCCATGCTCAGCTAC 2350
H Q F S H F L Y S N L Y P M L S Y

TCATCGGTCATCCTAGTATTGCGCCCTGATGTTTGAGAGACCAATATATA 2400
S S V I L V L R P D V

CAGTAAATAATATGTCTATATGTAGTCCTTTGGCGTATTATCAGTGTGCA 2450

CAATTGCTCTATTGCCAGTGATCTATTCGATCCACAGATACATGTGCAAA 2500

CTGCAAAGTTTCTGGTAATCAGAGAAGTTTTTCCTGAAAAAAAAAAAAAA 2550

AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA 2590

To date, various components essential for chloroplast protein import machinery have been identified (Hirsch et al., 1994, Schnell et al., 1994, Soll and Tien, 1998). One system that participates in translocation of starch synthetic enzymes into plastids involves phosphorylation by serine or threonine protein kinases (Waegemann and Soll, 1996). Waegemann and Soll (1996) proposed a phosphorylation motif $(P/G)X_n(R/K)X_n(S/T)X_n(S^*/T^*)$ for chloroplast precursor proteins, where n = 0-3 and S*/T* represents the phosphate acceptor. However, this motif was not observed within the predicted region of wheat isoamylase transit peptide but rather located downstream of the cleavage site of isoamylase transit peptide (Figure 4.9). The analysis suggests that the phosphorylation motif that occurred in chloroplast proteins may not be highly conserved in protein targeted to amyloplasts and/or phosphorylation at the site other than within the transit peptide region may also trigger the import machinery on amyloplast membrane of wheat. Alternatively, translocation of wheat isoamylase into amyloplast may be facilitated by other transport system.

Transit peptides of starch synthetic enzymes from wheat were compared using Megalign program (Figure 4.10). The results revealed that the transit peptide of wheat isoamylase showed only 22%, 14%, 24%, 26%, 27%, 22%, and 18% similarity to the transit peptides of GBSSI (Clark et al., 1991, Ainsworth et al., 1993), GBSSII (Vrinten and Nakamura, 2000), SBEI (Repellin et al., 1997), SBEII (Nair et al., 1997), SSI (Peng et al., 2001), SSII (Gao and Chibbar, 2000), and SSIII (Li et al., 2000) from wheat, respectively. On the other hand, the transit peptides of SBEIs from wheat (Repellin et al., 1997) and

maize (Fisher et al., 1995) showed 48% similarity. Therefore, the results suggest that transit peptides are not conserved based on species but rather conserved within the same gene regardless of the organism sources. Since the transit peptides of plant isoamylases have not yet been determined, it remains to be shown whether transit peptides of isoamylase are highly conserved regardless of the plant species. However, comparison of deduced amino acid sequences of isoamylases from maize (James et al., 1995) and wheat showed that the amino acid residues at the N-termini of wheat and maize proteins varied dramatically. Unlike those of SBEI, transit peptides of isoamylase from various sources might not show high sequence similarity.

According to the deduced amino acid sequence of mature wheat isoamylase, the polypeptide chain contains four conserved regions of isoamylase located in the middle region as deduced from nucleotide 1043 to 1657) (Figure 4.9). The wheat protein also carries two starch debranching enzyme conserved sequence blocks and eight isoamylase-specific conserved sequence regions (Figure 4.9) (Beatty et al., 1999). Among isoamylases from higher plants, wheat isoamylase showed 86%, 82%, and 81% simiarity to barley (GenBank accession number AF142589), rice (GenBank accession number AB015615), and maize (GenBank accession number U18908) isoamylases, respectively. A phylogenetic tree constructed from debranching enzymes from various sources confirmed that the isolated wheat cDNA belonged to isoamylase-type debranching enzyme (Figure 4.11). The DNAstar software showed that amino acid sequence similarity between wheat isoamylase and

pullulanases or glycogen debranching enzymes from various sources was only 12-13% and 9-10%, respectively.

In conclusion, the 2,590-bp cDNA isolated from the developing wheat kernel cDNA library contains in-frame start codon, possible cleavage site of transit peptide, conserved regions of isoamylase, stop codon, and poly-A tail. Comparison of nucleotide and deduced amino acid sequences confirmed the high similarity between isoamylases from wheat and other organisms. The size of the isolated cDNA is also similar to that of a transcript isolated from developing wheat kernels (Figure 4.14). Therefore, the 2,590-bp cDNA likely represents a full-length isoamylase cDNA in developing wheat kernels.

Percent Similarity

GBSS-I	*GBSS-II*	*SBE-I*	*SBE-II*	*SS-I*	*SS-II*	*SS-III*	*ISA*	
***	16.2	18.0	18.5	19.5	18.6	14.9	21.6	***GBSS-I***
	***	11.5	9.3	17.1	10.2	14.9	13.7	***GBSS-II***
		***	18.5	19.5	18.6	21.3	23.5	***SBE-I***
			***	19.5	16.7	24.1	25.5	***SBE-II***
				***	24.4	17.1	26.8	***SS-I***
					***	16.9	21.6	***SS-II***
						***	17.6	***SS-III***
							***	***ISA***

Figure 4.10 Comparisons of transit peptides from wheat
The diagram shows %similarity between various transit peptides of wheat starch synthetic enzymes; GBSSI (Clark et al., 1991, Ainsworth et al., 1993), GBSSII (Vrinten and Nakamura, 2000), SBEI (Repellin et al., 1997), SBEII (Nair et al., 1997), SSI (Peng et al., 2001), SSII (Gao and Chibbar, 2000), SSIII (Li et al., 2000), and isoamylase (ISA, this study). The similarity was obtained by comparison of deduced amino acid sequences in the transit peptide regions using Megalign program (DNAstar software).

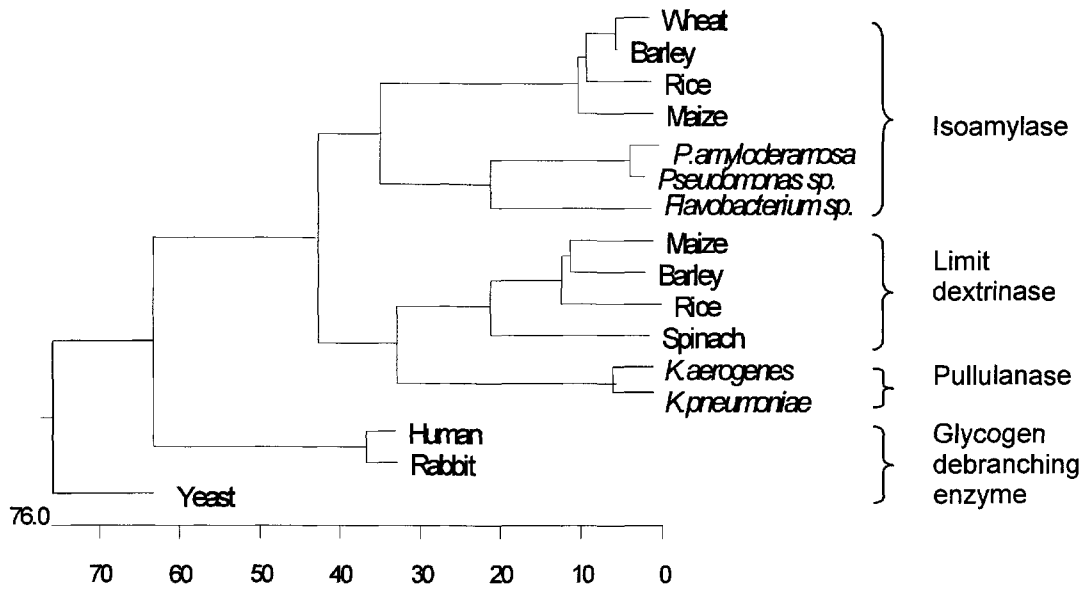


Figure 4.11 Phylogenetic tree of debranching enzymes
The tree was created from deduced amino acid sequences of debranching enzymes from various sources using the MegAlign program (DNAstar software). Isoamylases were obtained from kernels of wheat (this study), barley (AF142589), rice (AB015615), and maize (U18908), *Pseudomonas amyloderamosa* (J03871 M28370), *Pseudomonas sp.* (A10909), and *Flavobacterium sp.* (U90120). Plant limit dextrinases originated from spinach leaf (X83969), and endosperm of maize (AF080567), barley (AF022725), and rice (D50602). Bacterial pullulanases were obtained from *Klebsiella aerogenes* (M16187) and *Klebsiella pneumoniae* (X52181 M32702). The sources of glycogen debranching enzymes were human (M85168), rabbit (L10605), and yeast (AB018078). The tree shows that each type of debranching enzymes is more related to each other regardless of the organism source.

4.4 Expression of wheat isoamylase in *E. coli*

Wheat isoamylase was expressed in *E. coli* to analyze its enzymatic activity and to use as a source for polyclonal antibody production in rabbits. The pBluescript vector carrying a 2,590-bp wheat isoamylase cDNA was digested with NotI and XhoI (Figure 4.12). The wheat cDNA fragment was ligated to NotI-XhoI-treated pET-28(a) vector. The resulting recombinant plasmids of 8.0-kb were transformed into *E. coli* BL21(DE3). Recombinant isoamylase was expressed as a fusion protein carrying histidine- and T_7-tagging sequences at the N-terminus. Expression of recombinant protein was induced by addition of 1 mM IPTG. The recombinant protein could be observed on SDS-PAGE as a 92-kDa polypeptide. Coomassie staining of the gel showed that the 92-kDa polypeptide was exclusively accumulated in insoluble fraction of BL21(DE3) cells treated with IPTG (Figure 4.13A). Western blot analysis using T_7-tag antibody (Novagen) showed that the antibody recognized only the 92-kDa polypeptide present in the insoluble fraction of IPTG-treated cells (Figure 4.13B). The 92-kDa protein was then used as a source to produce polyclonal antibodies in rabbits. The rabbit anti-wheat isoamylase antibody recognized a polypeptide identical to that detected by T_7-tag antibody (Figure 4.13C). The results therefore suggested that the rabbit antibody specifically recognized recombinant protein encoded by the 2,590-bp cDNA from developing wheat kernels.

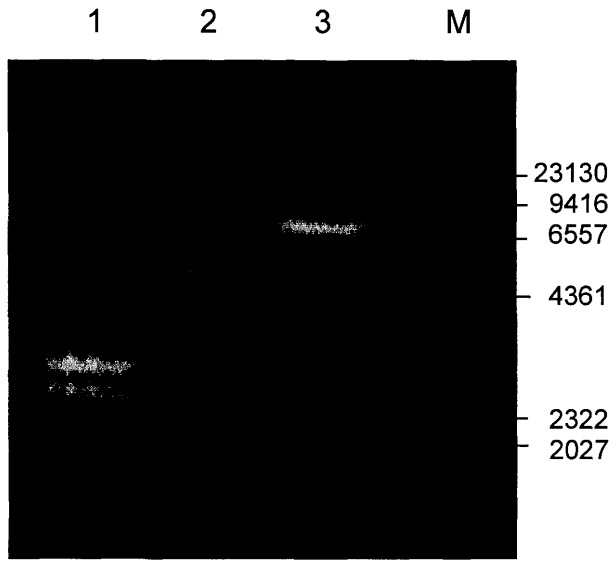


Figure 4.12 Isoamylase expression construct
The pBluescript carrying a 2,590-bp wheat isoamylase cDNA was digested with NotI and XhoI. Two DNA fragments, 3.0-kb pBluescript and 2.6-kb wheat isoamylase cDNA, were observed (lane 1). The 2.6-kb wheat cDNA fragment was ligated to NotI-XhoI-treated pET-28(a) vector (lane 2). The recombinant plasmids of 8.0-kb (lane 3) were transformed into *E.coli* BL21(DE3). The sizes in base pairs of molecular weight marker (M) are indicated on the right.

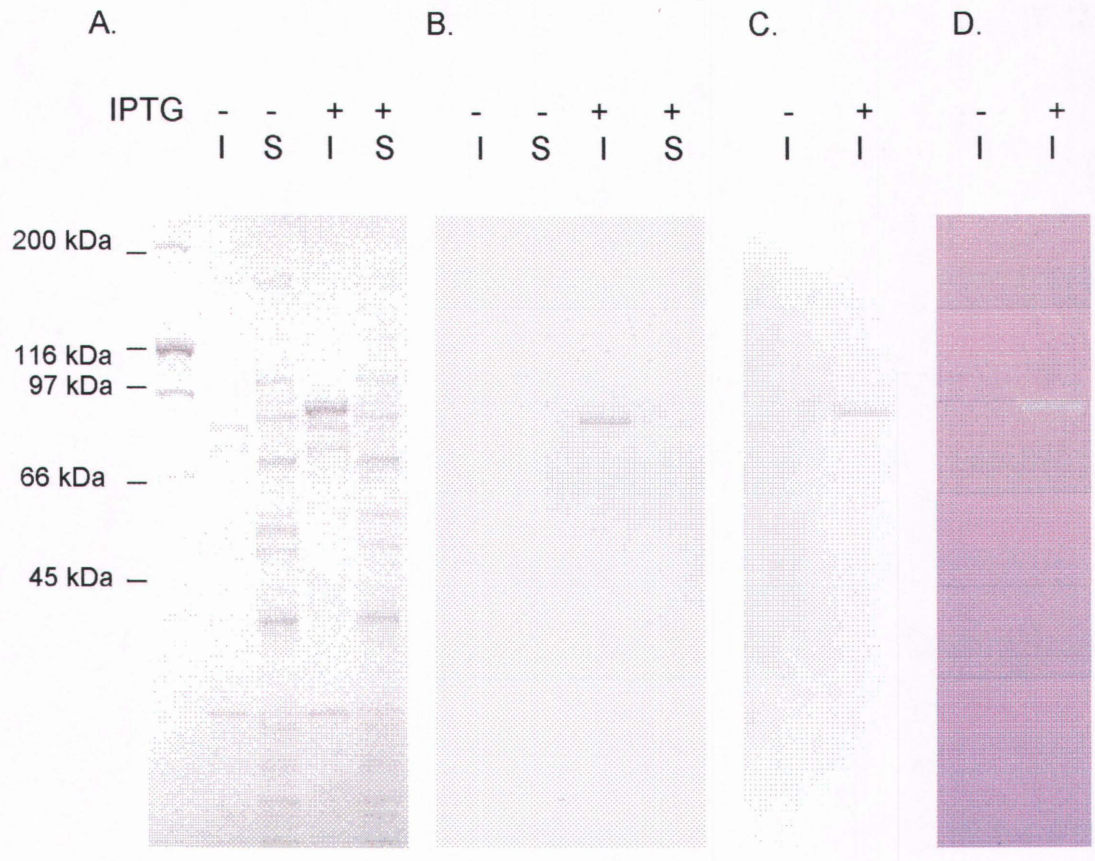


Figure 4.13 Expression of recombinant isoamylase in E. coli
Wheat isoamylase was expressed in *E. coli* as a 92-kDa fusion protein carrying T_7-tagging sequence at the N-terminus. Proteins from bacterial extracts were fractionated on starch-containing polyacrylamide gel and stained with Coomassie blue (A), and immunoblotted with T_7-tag antibody (B) and rabbit anti-wheat isoamylase antibody (C). Starch debranching activity of the recombinant protein was observed as a blue band on a purple background (D). Polypeptide profiles extracted from soluble (S) and insoluble (I) fractions of the cells were compared before (-) and after (+) addition of IPTG.

Zymogram analysis (Mouille et al., 1996) was performed to confirm starch debranching enzyme activity of the recombinant protein. This technique simultaneously shows the apparent molecular mass and enzymatic activity on a substrate-containing polyacrylamide gel. Proteins extracted from *E. coli* BL21(DE3) cells were fractionated on starch-containing SDS-PAGE gel. After electrophoresis, the polypeptides were renatured by overnight incubation of the gel in a Tris-glycine buffer (Mouille et al., 1996). Starch debranching enzyme activities could be observed by staining the gel with iodine. Hydrolysis of starch with starch debranching enzymes results in the formation of α-1,4-linked glucans. These linear chains form blue complex with iodine, while the branched polysaccharides of starch are stained purple. Therefore, localization of a starch debranching enzyme could be observed as the presence of a blue band on a purple background. The zymogram results showed that a single blue band was observed in BL21(DE3) treated with IPTG (Figure 4.13D). The position of the blue band corresponds with the 92-kDa polypeptide on starch-containing gel stained with Coomassie blue (Figure 4.13A). When proteins on starch-containing polyacrylamide gel were electroblotted onto a red pullulan-containing gel, a clear band could not be observed at the same relative mobility. Therefore, the 92-kDa protein showed isoamylase-typed debranching enzyme activity. The zymogram result implied that the catalytic activity of wheat isoamylase is carried out by one type of polypeptide chain. However, wheat isoamylase might function *in vivo* as a homo-multisubunit enzyme as observed in rice (Fujita et al., 1999).

4.5 Expression patterns of wheat isoamylase

4.5.1 In developing kernels

Northern blot analysis was used to study accumulation of isoamylase transcripts in 5, 10, 15, and 20 dpa wheat kernels. The 619-bp wheat isoamylase DNA fragment, labeled with DIG-dUTP, was used as a probe. The probe recognized a 2.6-kb transcript (Figure 4.14), whose size correlates with the 2,590-bp isoamylase cDNA isolated from the cDNA library (Figure 4.9). During wheat kernel development, the highest amount of isoamylase transcripts was observed in 5-dpa kernels. The transcripts gradually decreased as the grains matured. After the signals using isoamylase DNA probe were detected and the isoamylase DNA probe was removed, the RNA was re-hybridized with a 620-bp cDNA fragment of 25S rRNA (Molnar et al., 1989), labeled with DIG-dUTP. This step was to ensure that all the samples contained relatively the same amount of RNA. However, it did not confirm whether the mRNA pools in different samples were similar. Unlike a standard system used in animals where, for example, stable accumulation of an actin transcript is used as a control, a housekeeping gene is not available in plants. The transcript of Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase) large subunit is often used as a control in plant systems (Nassuth et al., 2000). However, because the Rubisco large subunit is encoded on chloroplast genome (Hartman and Harpel, 1994), this control system could be applied only to photosynthetic tissues. In this study, comparison of a ribosomal RNA is the only method to estimate equal amounts of RNA in different samples.

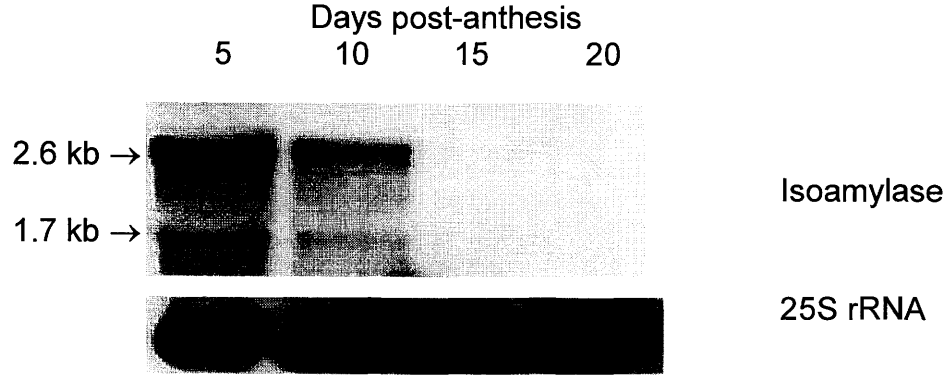


Figure 4.14 Accumulation of isoamylase transcripts in developing wheat kernels

Ten μg of RNA from 5, 10, 15, and 20 dpa wheat kernels were hybridized with the DIG-labeled 619-bp isoamylase DNA probe. Quality and quantity of RNA from different samples were compared by re-hybridization of the membrane with 25S rRNA probe.

In addition to the 2.6-kb isoamylase transcript, the probe also detected accumulation of a 1.7-kb transcript in 5-dpa wheat kernels. Accumulation of the 1.7-kb transcript during wheat kernel development closely followed the accumulation of the larger transcript. After the membrane was re-probed with 620-bp cDNA fragment of 25S rRNA, labeled with DIG-dUTP, the result showed that the amounts of RNA from 15- and 20-dpa kernels were slightly higher than those from 5- and 10-dpa kernels. However, the presence of 1.7-kb transcript could not be detected during those stages. According to the 1.5-kb wheat cDNA isolated from a cDNA library, different length at the 3′-region would likely result in the production of two isoamylase transcripts in developing wheat kernels. Therefore, 3′RACE was performed using RNA extracted from 5-dpa kernels as the template. Characteristics of the 1.7-kb wheat isoamylase transcript will be discussed in section 4.7.

Rahman et al. (1998) reported that isoamylase was exclusively localized in the soluble fraction of developing maize kernels. Therefore, soluble proteins from wheat kernels were extracted and subjected to immunoblot analysis using polyclonal antibodies against wheat isoamylase. The antibodies recognized a single polypeptide of approximately 83 kDa (Figure 4.15), which is similar to the apparent molecular mass predicted from the cDNA (Figure 4.9). It was further shown that accumulation of isoamylase in developing wheat kernels increased as the grains matured from five to 15 dpa. The polypeptide started to decrease in 20-dpa kernels until the antibodies could not detect it in mature dry seeds.

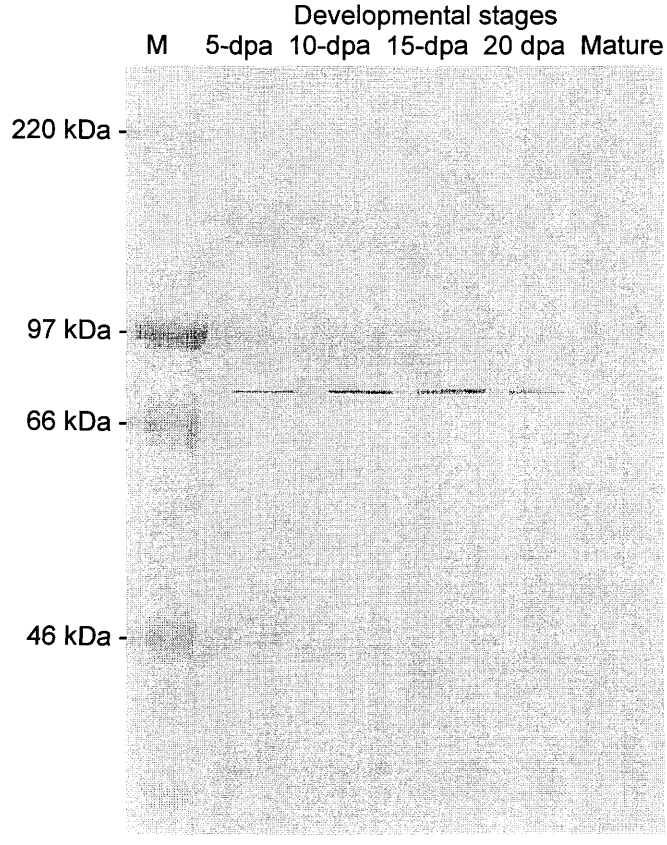


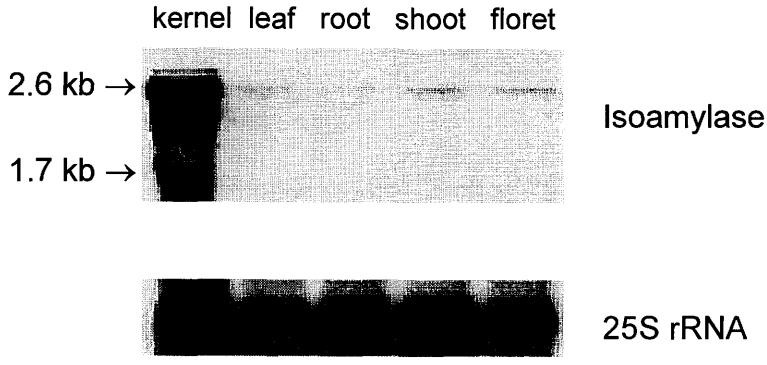
Figure 4.15 Immunoblot analysis of isoamylase in wheat kernels
Proteins were extracted from 5-, 10-, 15-, 20-, and mature wheat kernels. The proteins were fractionated on a polyacrylamide gel, and then subjected to immunoblot analysis using rabbit anti-wheat isoamylase antibodies.

4.5.2 In non-storage tissues

RNA extracted from leaf, root, shoot, and floret tissues of wheat was subjected to Northern blot analysis. The amount of isoamylase transcripts in non-storage tissues was compared with that in 5-dpa kernels. The 619-bp isoamylase DNA probe, labeled with DIG-dUTP, recognized a 2.6-kb transcript in all the tissues tested (Figure 4.16A). As compared to that in 5-dpa kernels, small amount of the transcript was observed in leaf, root, shoot, and floret tissues. In addition, the 1.7-kb transcript could not be detected in tissues other than in 5-dpa kernels. This may due to small amount of transcript accumulation in non-storage organs of wheat.

Results from RT-PCR confirmed that small amount of isoamylase transcripts was present in leaf, root, shoot, and floret tissues (Figure 4.16B). Amplification of a 315-bp fragment of 18S rRNA was used as an internal control for RT-PCR of various samples. The RT-PCR conditions were optimized to ensure that the desired product was amplified within a linear range. The number of PCR cycles was also minimized to avoid amplification saturation. Using RT-PCR alone might lead to question whether the amplified products result from a leaky expression of the gene. However, a combination of RT-PCR and Northern blot analysis would be able to confirm that wheat isoamylase was expressed at relatively low level in non-storage tissues as compared to that in developing grains.

A.



B.

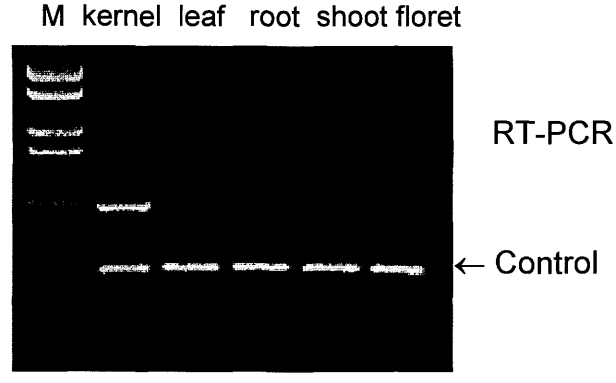


Figure 4.16 Spatial accumulation of isoamylase transcripts

A. RNA from 5-dpa kernels, leaf, root, shoot, and floret tissues was hybridized with the 619-bp wheat isoamylase DNA probe, labeled with DIG-dUTP. Quality and quantity of RNA from different samples were compared by re-hydbridization of the membrane with 25S rRNA probe.

B. RNA from wheat tissues was amplified using primers specific to the first and fourth conserved regions of isoamylase. Amplification of wheat isoamylase cDNA yielded a 619-bp product. Amplification of a 315-bp fragment of 18S rRNA was used as an internal control for RT-PCR. The molecular weight marker (M) contains 2176, 1766, 1230, 1033, 653, 517, 453, 394, 298, 234, 220, and 154 bp DNA fragments.

According to Sun et al. (1999), spatial expression of barley isoamylase is highly specific to endosperm tissue. Since barley and wheat are genetically related, the results observed from barley and wheat should not be dramatically different. Due to small amount of wheat transcripts present in non-storage tissues, the different expression patterns of wheat and barley isoamylases may result from the sensitivity of Northern blot analyses rather than the absence of the transcript in barley. In wheat, a small amount of starch is accumulated in non-storage tissues. This correlates with the presence of small amount of isoamylase transcripts in leaf, root, shoot, and floret tissues. The results has therefore suggested the involvement of wheat isoamylase in starch synthesis in non-storage organs. In leaf and floret tissues, the presence of isoamylase transcript may represent starch-degrading activity of isoamylase that functions in mobilization of starch during the dark period. However, because the plant materials used in this study were collected during the mid-day period, detection of wheat isoamylase transcript in leaf and floret tissues would rather indicate the starch synthetic activity of isoamylase.

4.5.3 In germinating seeds

In combination with amylases, starch debranching enzymes are involved in complete hydrolysis of starch during seed germination. As compared to the 2.6-kb transcript observed in developing wheat grains, the transcript of similar size was also observed in germinating wheat kernels (Figure 4.17). The levels of 2.6-kb transcript observed in wheat grains after 1 to 5 days of water imbibition were not significantly different. However, it seemed that the 2.6-kb isoamylase transcript was present at a slightly higher amount in seeds treated with 2 days of water imbibition and the transcript decreased with prolonged imbibition. As compared to the Northern signals observed in developing wheat kernels, detection of the isoamylase transcript in germinating seeds requires a prolonged exposure of the membrane. Therefore, the results indicated that compared with the transcripts accumulated during wheat kernel development, smaller amount of isoamylase transcript was observed during germination. The absence of the 1.7-kb transcript in germinating seeds may also result from the low expression of isoamylase gene.

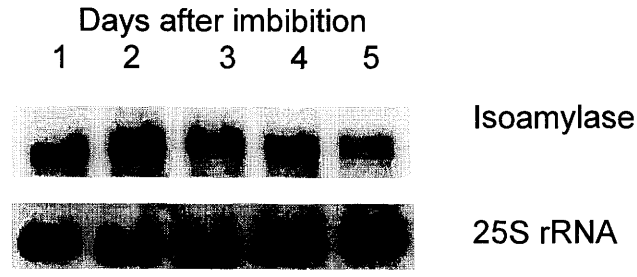


Figure 4.17 Accumulation of isoamylase transcript in germinating wheat grains

RNA was extracted from wheat seeds after 1 to 5 days of water imbibition. The DIG-labeled 619-bp fragment was used as a probe. The 2.6-kb isoamylase transcript was detected in every stage during germination. Quality and quantity of RNA from different samples were compared by re-hydbridization of the membrane with 25S rRNA probe.

4.6 Copy number of isoamylase gene in the wheat genome

Isoamylase participates both in starch breakdown during seed germination and in starch synthesis during cereal grain development. Southern blot analysis of wheat genomic DNA was performed to determine the gene copy number encoding the isoamylase isoforms responsible for these two distinct functions. The genomic DNA from freeze-dried leaves of *Triticum aestivum* L. cv. CDC Teal was digested with various restriction endonucleases. Based on the restriction maps of isoamylase genomic sequences from maize (Beatty et al., 1997, GenBank accession number AF030882) and barley (GenBank accession number AF142588), the maize and barley genomic DNA sequences do not contain restriction sites for ClaI, MluI, NotI, PvuI, SalI, and XhoI. These enzymes were also predicted not to cut the isoamylase gene in wheat. In Southern blot analysis, the 2,590-bp wheat isoamylase cDNA, labeled with DIG-dUTP, was used as a probe to ensure that all the DNA fragments could be identified.

If isoamylase activity in wheat is controlled by a single copy gene, and if the restriction enzymes do not cut within the gene, mutations that occur along the genome would therefore allow a maximum of three DNA fragments (one allele from each genome) to be detected by Southern analysis. In this study, only a single high molecular weight DNA fragment was observed after digestion of wheat genomic DNA with each restriction endonuclease (Figure 4.18). The results indicated that the isoamylase gene exists on only one genome of hexaploid wheat or, alternatively, the high molecular weight DNA fragment, as

visualized from Southern blot analyses, may be composed of three high molecular weight DNA fragments that are in the same relative mobility. If the isoamylase gene exists as a triplicated set of a single copy, one set present on each genome, detection of a single high molecular weight DNA fragment (Figure 4.18) would likely result from the low mutation frequency within the regions detected by Southern blot analysis. Therefore, this remains to be proven whether one isoamylase allele would exist on each genome of hexaploid wheat. No matter the isoamylase gene is present only a single allele on three genomes of wheat or one allele on each genome, the results shown here suggested the presence of a single isoamylase gene on the genome of a hexaploid wheat (*T.aestivum* L. cv. CDC Teal).

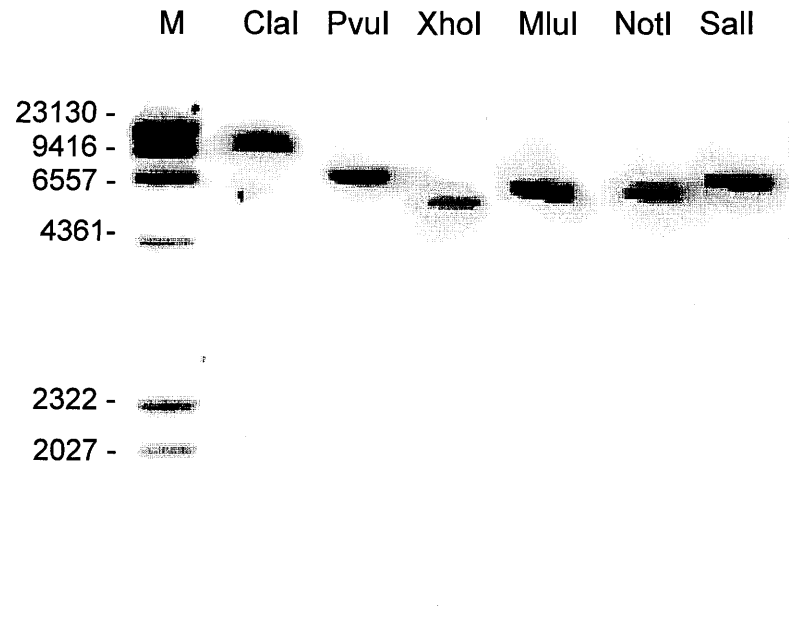


Figure 4.18 Southern blot analysis
Wheat genomic DNA extracted from freeze-dried leaves was digested with ClaI, PvuI, XhoI, MluI, NotI, and SalI in separate reactions. The DNA was hybridized with the DIG-labeled 2,590-bp wheat isoamylase cDNA. The sizes in base pairs of molecular weight marker (M) are indicated on the left.

4.7 Characteristics of a truncated wheat isoamylase transcript

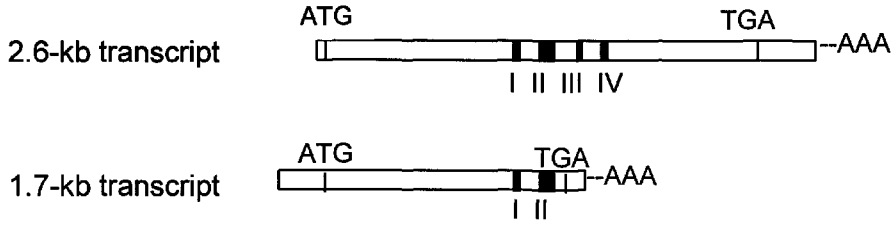
From the Northern analysis of isoamylase in developing wheat grains, the 619-bp probe also recognized a smaller transcript of approximately 1.7 kb (Figure 4.14). Based on the restriction maps of cDNA clones isolated from the developing wheat kernel cDNA library (Nair et al., 1997), a clone carrying 1.5-kb wheat cDNA showed a different 3′-region as compared to the other 19 clones isolated. The nucleotide sequence of the 1.5-kb cDNA fragment was determined and the result showed that as compared to the 2,590-bp wheat isoamylase cDNA the truncated cDNA has a longer 5′-untranslated region (101 bp) and most of the 3′-region is absent (Figure 4.19). However, nucleotide sequences in the common regions between the full-length and truncated cDNA are identical. The poly-A tail of the truncated isoamylase cDNA is located downstream of the second conserved region of isoamylase, thus producing a 51-kDa polypeptide containing only the first two conserved regions of isoamylase. In the 1.5-kb wheat cDNA, the presence of a poly-A tail may result from an alternate polyadenylation signal present in the intron regions.

The *in vivo* presence of the 1.7-kb transcript was confirmed using 3′-rapid amplification of cDNA ends (RACE) (Frohman et al., 1988) of total RNA extracted from 5-dpa wheat kernels. Amplified DNA fragments were cloned and sequenced. The results confirmed that the full-length and truncated wheat isoamylase transcripts carried variable 3′-regions. Expression of the 1.5-kb wheat isoamylase cDNA in *E. coli* produced a 54-kDa fusion protein. Although the polypeptide could react with rabbit anti-wheat isoamylase antibodies, its

starch debranching enzyme activity could not be observed on starch-containing polyacrylamide gel (Mouille et al., 1996).

Two isoamylase transcripts with different size were also observed in maize (Rahman et al., 1998) and barley (Sun et al., 1999). However, only the characteristics of those in barley were reported. Similar to wheat, the smaller isoamylase transcript of barley contains a shorter 3′ region, thus, coding for a truncated isoamylase protein. However, the smaller transcript of barley encodes the polypeptide with all four conserved regions of isoamylase. On the other hand, the truncated wheat isoamylase as deduced from the 1.5-kb cDNA lacks the third and fourth conserved regions of isoamylase. Therefore, the polypeptide is likely non-functional *in vivo* and is expected to be degraded after being recognized by a cellular protein degradation mechanism.Similar to isoamylase, two types of limit dextrinase transcripts were observed in developing kernels of maize (Beatty et al., 1999). Therefore, expression of starch debranching enzyme genes in developing cereal grains commonly results in the production of several transcripts.

A.



B.

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CTCCCACGAAGAACCAACAGGAGGCGCGGATCCCACCGATAAATAACCCC      50
GCCTCGCCGCTCCTCCCCAAAATCAATCACCGATCGCTCGGGGTTCCCGG     100
CATGACAATGATGGCCATGCCCGAGGCGCCCTGCCTCTGCGCGCGCCCGT     150
CCCTCGCCGCGCGCGCGAGGCGGCCGGGGTCGGGGCCGGCGCCGCGCCTG     200
CGACGGTGGCGACCCAATGCGACGGCGGGGAAGGGGGTCGGCGAGGTGTG     250
CGCCGCGGTTGTCGAGGCGGCGACGAAGGTAGAGGACGAGGGGGAGGAGG     300
ACGAGCCGGTGGCGGAGGACAGGTACGCGCTCGGCGGCGCGTGCAGGGTG     350
CTCGCCGGAATGCCCGCGCCGCTGGGCGCCACCGCGCTCGCCGGCGGGGT     400
CAATTTCGCCGTCTATTCCGGCGGAGCCACCGCCGCGGCGCTCTGCCTCT     450
TCACGCCAGAAGATCTCAAGGCGGATAGGGTGACCGAGGAGGTTCCCCTT     500
GACCCCCTGATGAATCGGACCGGGAACGTGTGGCATGTCTTCATCGAAGG     550
CGAGCTGCACAACATGCTTTACGGGTACAGGTTCGACGGCACCTTTGCTC     600
CTCACTGCGGGCACTACCTTGATGTTTCCAATGTCGTGGTGGATCCTTAT     650
GCTAAGGCAGTGATAAGCCGAGGGGAGTATGGTGTTCCAGCGCGTGGTAA     700
CAATTGCTGGCCTCAGATGGCTGGCATGATCCCTCTTCCATATAGCACGT     750
TTGATTGGGAAGGCGACCTACCTCTAAGATATCCTCAAAAGGACCTGGTA     800
ATATATGAGATGCACTTGCGTGGATTCACGAAGCATGATTCAAGCAATGT     850
AGAACATCCGGGTACTTTCATTGGAGCTGTGTCGAAGCTTGACTATTTGA     900
AGGAGCTTGGAGTTAATTGTATTGAATTAATGCCCTGCCATGAGTTCAAC     950
GAGCTGGAGTACTCAACCTCTTCTTCCAAGATGAACTTTTGGGGATATTC    1000
TACCATAAACTTCTTTTCACCAATGACAAGATACACATCAGGCGGGATAA    1050
AAAACTGTGGGCGTGATGCCATAAATGAGTTCAAAACTTTTGTAAGAGAG    1100
GCTCACAAACGGGGAATTGAGGTGATCCTGGATGTTCGTGTTCAAGCAGAC   1150
AGCTGAGGGTAATGAGAATGGTCCAATATTATCATTTAGGGGGGTCGATA    1200
ATACTACATACTATATGCTTGCACCCAAGGGAGAGTTTTATAACTATTCT    1250
GGCTGTGGGAATACCTTCAACTGTAATCATCCTGTGGTTCGTCAATTCAT    1300
TGTAGATTGTTTAAGATACTGGGTGACGGAAATGCATGTTGATGGTTTTTG   1350
GTTTTTTATTTTTTCATTCATTATGACCAGAGGTTCCAGTCTGTGGGATCCA  1400
GTTAACGTGTATGGAGCTCCATGAGAGACCAATATATACAGTAAATAATA    1450
TGTCTATATGTAGTCCTTTGGCGTATTATCAGTGTGCACAATTGCTCTAA    1500
AAAAAAAAAAAAAAAAA                                     1517
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5. GENERAL DISCUSSION

This study focused on starch debranching enzymes in developing wheat kernels. The major objective of the project was to identify and characterize starch debranching enzymes in wheat in order to determine whether or not starch debranching enzymes are involved in wheat starch synthesis. In this study, both types of starch debranching enzymes could be observed in developing wheat kernels. Since isoamylase activity could not be accurately determined from plant extracts, Western blot analysis using anti-wheat isoamylase antibodies was performed to study the presence of isoamylase in developing wheat grains. Although Western blot analysis can not elucidate whether isoamylase present in wheat grains is enzymatically active, the relationship between the concentrations of starch and the presence of limit dextrinase and isoamylase in developing wheat grains indicated that both types of starch debranching enzymes are involved in wheat starch synthesis.

Based on data obtained from *Arabidopsis* mutants lacking chloroplastic isoamylase, Zeeman et al. (1998) indicated that breakdown of leaf starch during the dark period was not affected by the loss of chloroplastic isoamylase. In addition to starch, the mutants accumulated chloroplastic phytoglycogen, which was also mobilized during the dark period. As compared to isoamylase, limit dextrinase has low activity toward phytoglycogen. The evidence observed in

Arabidopsis mutants has suggested that additional isoform(s) of isoamylase is (are) responsible for starch degradation. This speculation is possible because all starch synthetic enzymes known to date commonly contain more than one isoform encoded by a different gene or a member of a multi-gene family. In this study, Southern analysis of wheat genomic DNA showed that isoamylase exists as a single copy in the wheat genome. Therefore, if more than one isoform of wheat isoamylase exists *in vivo*, they could be produced through post-transcriptional modification, for example, using an alternative intron splicing mechanism (Båga et al, 1999b). Doehlert and Knutson (1991) identified two forms of maize isoamylase using anion exchange chromatography. However, polyclonal antibodies raised against recombinant maize isoamylase recognized only a single polypeptide chain (Rahman et al., 1998). Therefore, limited information on isoamylase isoforms is currently available. To date, deduced amino acid sequences of isoamylase cDNA isolated from various species have been classified into only one group. Zeeman et al. (1998) indicated that two isoamylase DNA sequences were found in the *Arabidopsis* genome; however, only one has a high similarity to maize isoamylase. Alternatively, additional isoamylase activity might be contributed from a distantly related gene.

Although two distinct models have been proposed to explain the role of starch debranching enzymes in amylopectin biosynthesis (Myers et al., 2000, Smith, 1999), we can not determine at present how these enzymes contribute distinctly or coordinately to amylopectin structure. It is known that limit dextrinase and isoamylase exhibit different substrate specificities and kinetic

properties for various glucans. These biochemical differences, therefore, result in different preference for modes of branching of polyglucans between limit dextrinase and isoamylase, and thus may indicate different accessibility of the enzymes to highly branched substrates *in vivo*. Doehlert and Knutson (1991) suggested that isoamylase appeared to be capable of readily hydrolysing less exposed α-1,6-branches, while limit dextrinase may hydrolyse exposed α-1,6-linkages more readily. Therefore, the hydrolytic activity of isoamylase is possibly essential to make tightly packed amylopectin molecules more accessible for limit dextrinase. In bacteria, the minimum number of branched molecules required for debranching by isoamylase differs from the number required by pullulanase (Lee and Whelan, 1971). This suggests that isoamylase and pullulanase play distinct roles in amylopectin biosynthesis, although it is possible that the two enzymes can complement the roles of the other to some extent (Kubo et al., 1999).

Unlike isoamylase, mutants with the primary lesion affecting limit dextrinase activity have not been identified in plants. This may be due to either the detrimental effect resulting from the lack of limit dextrinase or the incapability of the mutants to produce a variant starch phenotype. According to the widely characterized starch degrading activity of limit dextrinase in germinating grains of many plant species, limit dextrinase might function mainly in starch degradation rather than in starch biosynthesis. On the other hand, comparisons of the amounts of isoamylase transcripts in developing and

germinating grains of barley (Sun et al., 1999) and wheat (this study) have indicated the main *in vivo* function of isoamylase in starch biosynthesis.

Unlike the glycogen synthesis pathway, details of the starch synthesis pathway are not clearly understood. The complexity of the starch synthesis pathway is due to the complicated structure of starch itself and the existence of many isoforms of starch synthetic enzymes. For starch debranching enzymes, two types of enzymes with different substrate specificity also complicate the understanding of their roles in starch synthesis. However, the results shown here indicate that both types of starch debranching enzymes are involved in wheat starch biosynthesis. Further study is required to fully understand the starch synthetic activities of limit dextrinase and isoamylase in wheat.

6. CONCLUSIONS

- During wheat kernel development, the amount of starch accumulated in grains increased; however, a constant ratio of amylose and amylopectin was observed throughout developmental stages.
- Both types of starch debranching enzymes, limit dextrinase and isoamylase, were present in developing kernels of wheat (*Triticum aestivum L.* cv CDC Teal)
- Highest activity of limit dextrinase was observed in 15-dpa wheat kernels.
- A 2,590-bp cDNA fragment isolated from 12-dpa wheat kernel cDNA library encodes an 88-kDa polypeptide chain carrying a plastidal transit peptide of 5 kDa at the N-terminus.
- Expression of the 2,590-bp wheat cDNA in *E.coli* produced a recombinant protein with isoamylase-type starch debranching enzyme activity.
- Two isoamylase transcripts were observed during wheat kernel devlopment. As compared to the 2.6-kb isoamylase transcript, the 1.7-kb transcript lacks most of the 3′ region, and thus encodes truncated isoamylase that carries only the first two conserved regions of isoamylase. Starch debranching enzyme activity of the truncated isoamylase could not be detected in zymogram analysis.

- Isoamylase transcripts accumulated in developing and germinating wheat grains, as well as in leaf, root, shoot, and floret tissues.
- Western blot analysis using anti-wheat isoamylase antibodies detected a single polypeptide of 83-kDa in developing wheat kernels. The abundance of this polypeptide increased as the kernels developed from five to 15 dpa, and then started to decrease until it could not be detected in mature dry seeds.
- Isoamylase gene is present as a single copy in the genome of hexaploid wheat (*Triticum aestivum* L. cv. CDC Teal).

7. FUTURE RESEARCH

Two distinct roles of starch debranching enzymes have been indicated in cereals. These include starch degradation during seed germination and starch biosynthesis during kernel development. Study of limit dextrinase activity and molecular characterization of an isoamylase cDNA in developing wheat grains showed that both type of starch debranching enzymes are present during development of wheat kernels. This preliminary study of wheat starch debranching enzymes has therefore suggested a role of limit dextrinase and isoamylase in starch synthesis. Since the result from this study showed that isoamylase exists as a single copy in the genome of hexaploid wheat, molecular characterization of an isoamylase genomic DNA from wheat might reveal the factors controlling isoamylase gene expression during two very different developmental stages of wheat plants. Nucleotide sequence analysis of isoamylase genomic DNA from wheat might reveal the sequences that are involved in the production of transcripts with variable 3′ region. In addition, the isoamylase genomic DNA can also be used in promoter studies. The presence of more than one promoter at the 5′ region of the gene may result in the synthesis of various transcripts that encode for polypeptide chains with different catalytic activities (Båga et al., 1998). Besides the promoter sequences, the 5′-untranslated region of the gene may also contain sequences that mediate the

effects of exogenous signals. For example, the promoter region of a maize isoamylase gene contains two sequences (Schulze-Lefert et al., 1989, van der Steege et al., 1992) important for environmental induction of isoamylase gene expression (Beatty et al., 1997). In barley, a motif closely related to sucrose inducible sequence (Ishiguro and Nakamura, 1994) has been observed in the promoter region of the isoamylase gene and expression of the barley gene could be induced by exogenous sucrose (Sun et al., 1999). To date, it is not clearly known what factors control expression of isoamylase during two developmental stages of plants. However, regulation of gene expression at the transcriptional level is likely to be one of the factors.

In a few plant species, mutants lacking isoamylase activity have been used as models to study the effect of starch debranching enzymes on the structure and amount of starch. However, because of the hexaploid nature of the wheat genome (2n = 6x = 42; AABBDD), a wheat line with a null allele for starch debranching enzyme in its genome is unable to produce a variant starch phenotype. To understand a role of isoamylase in wheat starch synthesis, wheat lines with altered starch debranching enzyme activities must be produced using genetic engineering techniques. Wheat lines with reduced isoamylase activity could be obtained by introduction of the wheat cDNA, as characterized in this study, in an antisense orientation (Båga et al., 1999a). Expression of the antisense construct under the control of a seed specific promoter may result in the inhibition of isoamylase expression in wheat kernels. Consequently, the reduction or absence of isoamylase in the transgenic wheat kernels may result

in a variant starch phenotype. Characterization of these novel wheat lines would help us to understand the role of isoamylase in wheat starch synthesis.

The information obtained from the isoamylase cDNA from wheat can also be used to make genetic markers to screen wheat lines with isoamylase null allele. In addition to the application of genetic engineering techniques, wheat lines with reduced isoamylase activity could be produced by combining null alleles in all three genomes (Demeke et al., 1999). Comparisons of the amounts and structure of starch present in these mutant lines with those from wild type plants might indicate the effect of isoamylase on starch biosynthesis in wheat.

Unlike isoamylase, a mutant lacking limit dextrinase activity has not been observed in plants. At the moment, a cDNA encoding limit dextrinase in wheat is being characterized (Repellin et al., 1998). Similar to isoamylase, wheat lines with altered limit dextrinase activity could be produced. Study of limit dextrinase in wheat would further advance the understanding of wheat starch debranching enzymes. Combination of the information obtained from wheat and other plant species might reveal details in the starch biosynthesis pathway. After the starch biosynthesis pathway is thoroughly understood, plants with desired starch properties could be obtained through genetic modification.

Since the discovery of wheat mutants lacking a *waxy* protein (Yamamori et al., 1994) and their properties associated with the noodle-making industry (Miura and Tanii, 1994), a new perspective to produce novel starch has been given to Canadian wheat (Demeke et al., 1997). Identification of wheat with

novel starch properties might give Canadian wheat an opportunity in a new market place. As compared to the native starch, starch from wheat lines with altered starch debranching enzyme activities might exhibit different properties, and, thus, be suitable for a special end-use.

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